Effect-Based Analysis of Endocrine Disrupting Chemical Mixtures in Breast Milk and Possible Health Consequences for Human Infants.

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EFFECT-BASED ANALYSIS OF ENDOCRINE DISRUPTING CHEMICAL MIXTURES IN BREAST MILK AND POSSIBLE HEALTH CONSEQUENCES FOR HUMAN INFANTS.

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“Give me a child until he is 7 and I will show you the man.”

- Aristotle

Contents

List of Abbreviations

A

AAF: 2-acetamidofluorene

ACN: acetonitrile

AR: androgen receptor

atRA: trans-retinoic acid

B

BPA: bisphenol A

BFRs: brominated flame retardants

C

CALUX: Chemically Activated LUciferase eXpression

D

DART: developmental and reproductive toxicity

DBP: dibutylphthalate

DCC: charcoal-stripped fetal calf serum

DEHP: di-2-ethylhexyl phthalate

DES: diethylstilbestrol

DHEA: dehydroepiandrosterone

DHT: dihydrotestosterone

DON: deoxynivalenol

E

E1: estrone

E2: 17β-estradiol

E3: estriol

EACs: endocrine active compounds

EATS: estrogens androgens thyroid steroidogenesis

EDCs: endocrine disrupting chemicals

EPA: Environmental Protection Agency

ERα: estrogen receptor alpha

ERβ: estrogen receptor beta

EU-NETVAL: European Union Network of Laboratories for the Validation of Alternative Methods

G

GC: gas chromatography

H

H4PFOS: 1H,1H,2H,2H-perfluorooctanesulfonic acid

HBCD: hexabrominated cyclododecane

HCB: hexachlorobenzene

HPLC: high-performance liquid chromatography

HPLC-Q-TOF-MS/MS: high resolution quadrupole time-of-flight mass spectrometry

HRE: hormone-responsive elements

HSA: human serum albumin

HUMIS: human milk study

L

LC: liquid chromatography

LOECs: lowest observed effect concentrations

LOQ: limit of quantification

M

MeOH: methanol

N

NA: non-active

NEAA: non-essential amino acids

NTA: non-target analysis

O

OECD: Organization for Economic Co-operation and Development

P

P/S: penicillin streptomycin

PBP: pentabromophenol

PCBs: polychlorinated substances

PCP: pentachlorophenol

PFAS: perfluoroalkyl substances

PFBA: perfluorobutyric acid

PFBS: perfluorobutanesulfonic acid

PFDA: perfluorodecanoic acid

PFHpA: perfluoroheptanoic acid

PFHpS: perfluoroheptanesulfonic acid

PFHxA: perfluorohexanoic acid

PFHxS: perfluorohexanesulfonic acid

PFPeA: perfluoropentanoic acid

PFNA: perfluorononanoic acid

PFOA: perfluorooctanoic acid

PFOS: perfluorooctane sulfonic acid

PFOSA: perfluorooctanesulfonamide

POPs: persistent organic pollutants

Q

QuEChERS: quick easy cheap effective rugged and safe

R

REP: relative potency

RLUs: relative light units

RXR: retinoic acid receptor

S

SPE: solid phase extraction

T

T2-toxin: T-2 mycotoxin

T3: 3′,5,3-triiodo-[L]-thyronine; triiodothyronine

T4: 5′,3′,5,3-tetraiodo-[L]-thyronine; thyroxine

TCBPA: tetrachlorobisphenol A

TBBPA: tetrabromobisphenol A

TBG: thyroxine-binding globulin

TETRAC: 3,3'5,5'-tetraidothyroacetic acid

THs: thyroid hormones

TPO: thyroperoxidase

TRα: thyroid receptor alpha

TRβ: thyroid receptor beta

TRs: thyroid receptors

TREs: thyroid response elements

TRH: thyroid-releasing hormone

TRIAC: 3,3'5-triiodothyroacetic acid

TSH: thyroid-stimulation hormone

TTR: transthyretin

General Introduction

The research described in this thesis investigates the presence of endocrine disrupting chemicals (EDCs) in human breast milk samples, using extraction- and effect-based analytical methods. In addition, studies have been performed to investigate the chemical nature of the EDC activities, using bioassay directed fractionation and chemical identification methods. Furthermore, possible associations between the EDCs and infant health outcome such as cryptorchidism have been studied using samples and data from the Norwegian HUMIS (HUman MIlk Study) cohort.

Breast milk contamination and child adverse outcomes: the Norwegian HUMIS Cohort

As a natural source of nutrients and antibodies, breast milk is acknowledged to have many benefits for both infants and nursing mothers e.g. reduced risk of childhood diseases, infections, obesity, cardiovascular disease, and breast- and ovarian cancer (Ballard and Morrow 2013; Chowdhury et al. 2015; Sankar et al. 2015; Victora et al. 2016). Breastfeeding has always been highly encouraged and was declared as 'one of the most effective ways to ensure child health and survival' by the World Health Organization (WHO 2015). Over the past few decades, many studies further detailed the importance of exclusive breastfeeding during the first six months of life with regard to early infancy development and growth (WHO 2015). In parallel, a raising concern emerged from diverse toxicological studies presenting breastfeeding as a potential source of exposure to exogenous substances with possible endocrine active properties (Forns et al. 2015; Main et al. 2007; Massart et al. 2005; Sonawane 1995; Thomsen et al. 2010).

A variety of chemicals have been found to be able to interfere with some aspects of the endocrine system, e.g., estrogen-, androgen-, thyroid hormone action, which may result in the alteration of functions of the endocrine system and potential short- and long-term adverse effects in humans and wildlife species (Andersson et al. 2012; Colborn et al. 1993; Fucic et al. 2012; Schreiber et al. 2020). In 1996, a large international working group officially classified these hormonally active agents under the designation 'endocrine disrupting chemicals' (EDCs; IPCS 2002). Nowadays, these are defined as follows: 'An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations' (IPCS 2002).

To this day, many chemicals have been identified or suspected to be EDCs in experimental and wildlife studies although only a relatively limited number of them have been properly assessed with respect to human health effects and few are regulated (Bergman et al. 2012).

Even though studies highlighted the presence of potential EDCs in human milk, their origins and possible impacts on human health are still unclear (Andersson et al. 2012; Colborn et al. 1993; Iszatt et al. 2019; Kang et al. 2016; Solomon and Weiss 2002; Stefanidou et al. 2009). Since 2003, the population-based HUMIS (HUman MIlk Study) birth-cohort aims to investigate the impact of an early-life exposure on child health while identifying key factors contributing to human milk contamination (Eggesbø et al. 2009). For that purpose, the Norwegian Institute of Public Health collected 2606 breast milk samples from mother and child pairs originating from six Norwegian counties. Participating mothers were asked to fill in questionnaires at different time points (time of recruitment, after parturition at one, six, twelve, twenty-four months and seven years of age), providing information about lifestyle habits, breastfeeding patterns, and child's health outcomes including endocrine-dependent defects such as cryptorchidism i.e. undescended testis.

Subsequent chemical target analysis and case-studies showed the presence of environmental toxicants in HUMIS samples e.g. polychlorinated substances (PCBs), perfluoroalkyl substances (PFAS), brominated flame retardants, hexachlorobenzene (HCB), dioxins and dioxin-like compounds, and their possible association with health outcomes in children (Eggesbø et al. 2009; Forns et al. 2015; Iszatt et al. 2016). Although target investigations are of interest, EDCs can work additively, synergistically, or antagonistically and as a result, may lead to variable effects depending on the composition and concentration of the chemical mixtures. This implies that the sole investigation of individual compounds, or subclasses, such as persistent or non-persistent EDCs cannot provide enough information to accurately predict adverse outcomes derived from breastfeeding-based exposure (Heys et al. 2016; Kortenkamp 2014; Rajapakse et al. 2002). Therefore, there is a growing need of developing suitable assessment methods covering the combined effects of the total EDC load present in human milk.

EDC exposure: possible implication in endocrine-related diseases

Over the last decades, there has been a significant increase in the incidence of endocrine-related cancers e.g. thyroid, testicular, prostate, and sexual deformities in parallel with a drastic drop in fertility rates (Carlsen et al. 1995; Mascarenhas et al. 2012; United Nations Department of Economic and Social Affairs Population Division 2020; Wild et al.

2020). Throughout the past years, numerous pieces of evidence suggested the possible involvement of environmental toxicants, with endocrine disrupting properties, in the rising prevalence of hormonal-related diseases. While the role of genetics is not to be overlooked, the quick pace of these unprecedented changes affecting human health suggests that they cannot be purely attributed to inheritance factors.

In 1993, Sharpe and Skakkebaek showed that *in utero* exposure to the xenoestrogen diethylstilbestrol (DES) could affect male reproductive development leading to impaired fertility and a higher risk of endocrine cancer at adult age. Subsequently, many other toxicological studies revealed that EDCs, in particular estrogen-like and anti-androgenic substances, are strong actors in increasing male birth defects including cryptorchidism and hypospadias i.e. penile malformation (Bai et al. 2017; Gray et al. 2000; Sharpe and Skakkebaek 2003; Vinggaard et al. 2005b).

From fetal development to further maturation during infancy, childhood and puberty, the reproductive system highly depends on multiple endocrine actions (Marty et al. 2003; Müller and Skakkebaek 1992; Stiles and Jernigan 2010). For this reason, this system is a very sensitive target of potential mother-child EDCs exchanges, including placental transfer during fetal development and post-natal exposure through breastfeeding. Ethical and legal constraints are severely limiting the direct assessment of perinatal exposure to exogenous chemicals, therefore, identifying a suitable proxy for estimating EDCs activity during these critical periods is of major interest.

Breast milk: non-invasive matrix and invaluable source of information

Breast milk is a unique matrix, defined as a primary source of nutrition of many newborns and directly provided by women's breasts. It is the only non-invasive body fluid capable of offering information regarding the mother's chemical burden, including EDCs, which can also be a suitable proxy to her child's perinatal exposure conditions (Esteban and Castaño 2009). Due to its high lipid content, breast milk is often used for monitoring lipophilic pollutants such as persistent organic pollutants (POPs), known to distribute equally in all fat compartments of the body (Kanja et al. 1992; Waliszewski et al. 2001; WHO 2007). Considering their potential hormonal disrupting properties, POPs and other organic toxicants may be detrimental to the endocrine system, including reproductive functions (Gregoraszczuk and Ptak 2013; WHO 2007). Moreover, they tend to remain in the food chain and in fat-rich tissues where their concentration builds-up over lifetime. This bioaccumulation can constitute a problematic chemical body burden for future mothers and contributes to the development

of an unfavorable environment for the growing fetus. This exposure can potentially continue during infancy through transfer of the load of chemicals from mother to child via breastfeeding. To reach a better understanding of such an early-life exposure impact on a child's reproductive development, it is essential to identify key targets involved in environmental pollutants- and general EDC toxicity.

Sex steroids: key targets of EDCs-induced reprotoxicity

The action of natural hormones relies on their ability to navigate from their endogenous point of synthesis to their target tissues, where they can interact with specific endogenous receptors. Estrogens, i.e. estrone (E1), 17α-estradiol, 17β-estradiol (E2), and estriol (E3) bind with high affinity to nuclear estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) while androgens such as androstenedione, dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT) and testosterone, are natural ligands of the androgen receptor (AR). Following activation, these nuclear receptors form dimers that undergo translocation into the nucleus of the target cell, where they bind to specific hormone responsive elements on the DNA subsequently triggering the transcription of associated genes.

Over the past years, many classes of chemicals were proven to be able to replace natural hormones from their natural protein carriers and hormonal receptors. Because of their estrogen-like activities, several phthalates, insecticides, and flame retardants as well as natural phytoestrogens and mycotoxins were placed in the xenoestrogen sub-category of EDCs (Bergman et al. 2012). These toxicants are known to mimic the action of endogenous estrogens possibly leading to the uncontrolled activation, or suppression (in case of antagonists) of ERα and ERβ. Exposure to environmental xenoestrogens was shown to be potentially associated with various developmental abnormalities both in wildlife and in humans, including precocious puberty and feminization of the male reproductive tract (Fucic et al. 2012; Massart et al. 2008; Nikaido et al. 2004; Toppari et al. 1996).

In a similar manner, many chemicals may interfere with the androgen signaling pathway via AR binding, further disturbing the sensitive balance between estrogens and androgens levels (Kelce et al. 1998; Kelce and Wilson 1997). Various pesticides were also found to possess antagonistic (anti-) properties towards androgen action, e.g. linuron, fenarimol, procymidone, methoxychlor and prochloraz (Hotchkiss et al. 2004; Kelce et al. 1998; Van der Burg et al. 2010; Vinggaard et al. 2002, 2005b; Wilson et al. 2008). Furthermore, studies reported that natural estrogens as well as xenoestrogens may also act as potent anti-androgens (Sohoni and Sumpter 1998; Sultan et al. 2001; Vinggaard et al. 2005a). A multitude of *in vitro*

and *in vivo* evidence suggested that early exposure to those anti-androgenic substances may result in reproductive deformities including cryptorchidism and hypospadias as well as impaired fertility (Bai et al. 2017; Borch et al. 2006; Fisher 2004; Schreiber et al. 2020; Toppari et al. 1996; Tyl et al. 2004; Vinggaard et al. 2005a; Welsh et al. 2008).

As detailed earlier, there are numerous pieces of evidence demonstrating the critical role of estrogens and androgens in modulating the reproductive development, from fetal organs to full post-natal maturation. Hence, sex steroid signaling pathways, including AR and ERα/ERβ and their natural ligands, are key targets for studying potential reproductive impairments originating from perinatal EDC exposure.

EDC interferences with thyroid hormone transport: a direct access to the growing child

The thyroid signaling pathway relies on proper TH transportation from the thyroid gland to target cells where they can bind to nuclear thyroid receptor alpha (TRα) and thyroid receptor beta (TRβ) (Kim and Cheng 2013; Ortiga-Carvalho et al. 2014). THs are commonly transported by three major serum carrier proteins, thyroxine-binding globulin (TBG), transthyretin (TTR) and human serum albumin (HSA) (Pappa et al. 2015). Although it is well-known that THs major transporter is TBG, TTR is the only carrier capable to mediate in passage of THs over the blood-brain barrier and the uterine-placental wall making this protein the main carrier of THs in the cerebrospinal fluid and the developing fetus (Landers et al. 2013). 5′,3′,5,3-tetraiodo-[L]-thyronine (thyroxine; T_4) and its bioactive form 3′,5,3-triiodo-[L]-thyronine (triiodothyronine, T_3) are natural ligands capable of activating TRα/TRβ mono- or dimerization inducing their translocation to hormonal response elements, regulating thyroid-associated gene expression (Brent 2012). Moreover, thyroid receptors often interact with retinoid X receptor (RXR), a retinoic acid receptor, to form heterodimers (Hsu et al. 1995; Zhang et al. 1992).

In the past years, an increasing concern was raised regarding thyroid-disrupting effects of environmental toxicants. Highly persistent chemicals such as dioxins, PCBs and PFAS, were found to interfere with thyroid hormone metabolism at different biological levels e.g. decrease in THs circulating levels and/or interactions with TTR (Boas et al. 2012; Calsolaro et al. 2017). Rodent studies suggested that pollutants can transfer from the mother to the fetus compartment through the placenta by making use of TTR-binding to facilitate its transport, resulting in the reduction of T_4 levels in the offspring (Lau et al. 2003; Meerts et al. 2002; Thibodeaux et al. 2003). In 2009, Weiss et al., demonstrated the competitive binding

inhibition potency of PFAS compounds towards T_4 on TTR, contributing to the understanding of potential environmental toxicants' route of exposure from mother to child.

To this date, there is a real lack of more extensive studies investigating EDCs' placental transfer. Although this source of exposure mainly concerns the prenatal period, the lipophilic characteristic of most of thyroid-disrupting pollutants suggest that breast milk could represent a suitable proxy for further investigating early mother-child exposure.

Effect-based bioassays for the detection of hormone-disrupting activities

Mammalian reproduction and development, while being extremely complex, reuses a limited set of conserved pathways for different developmental processes. Therefore, it has been established that single specific *in vitro* effect-based bioassays covering only a few endpoints of these pathways may provide good predictions with regard to developmental and reproductive toxicity landmarks, such as sex organ deformities (Van der Burg et al. 2013). During the past decades, BioDetection Systems b.v. developed a large panel of highly specific and sensitive effect-based *in vitro* tests: the CALUX® (Chemically Activated LUciferase eXpression) reporter gene assays. These assays consist of mammalian cells, which are stably transfected to express a receptor of interest, e.g. ERα or AR, which upon ligand-binding transactivates receptor-specific gene expression, including the reporter gene luciferase derived from the firefly (Figure 1). In practice, cells are exposed to a dilution series of the test compound (pure chemical or sample extract) and incubated for about 24 hours. The next day, the substrate luciferin is added to the luciferase produced by the triggered cells resulting in light production. The produced light signal is proportional to the amount of ligand causing receptor activation and is quantified using a luminometer. The method is performed in both 96- to 384-well plate formats either by hand or using a robot-based automated setup, allowing high-throughput screening of many samples while only requiring a small volume of extract.

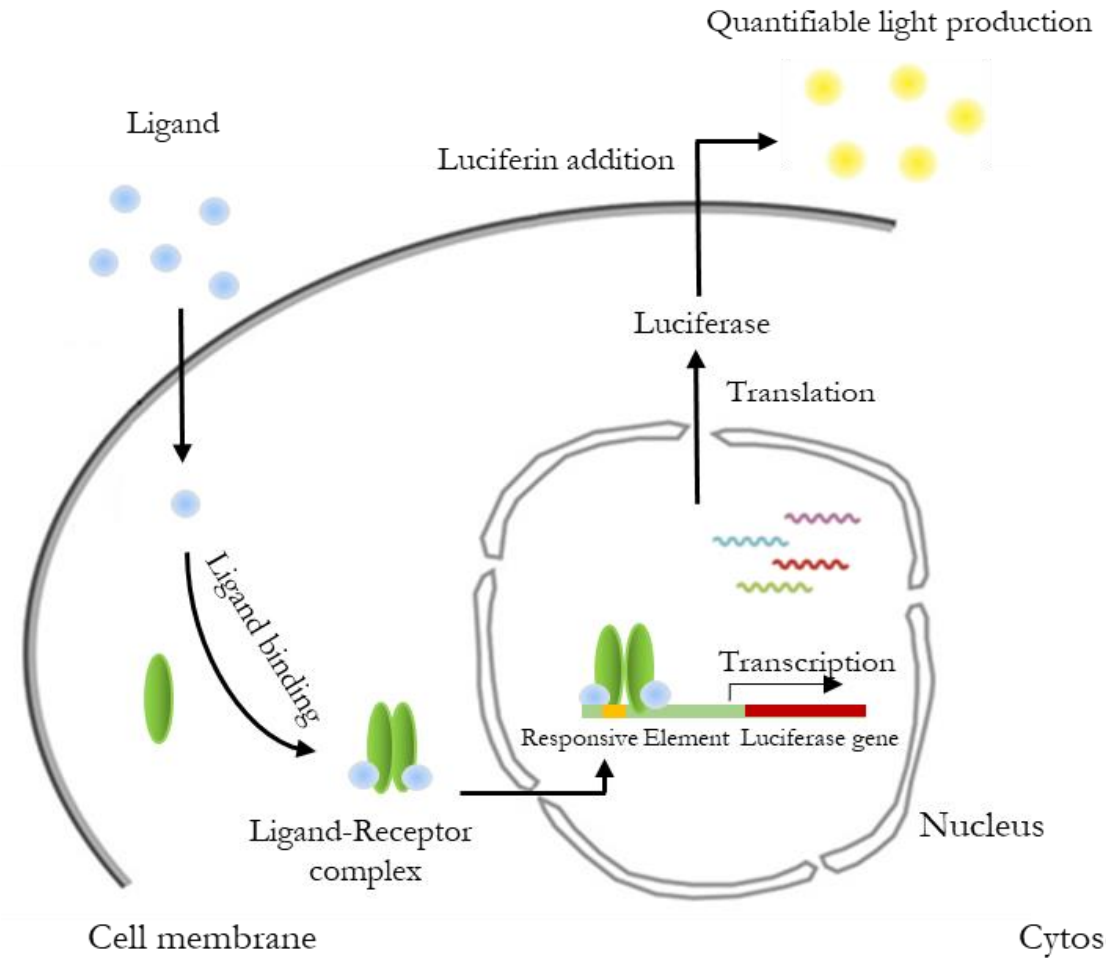

Figure 1. Schematic representation of Chemically Activated LUciferase gene eXpression (CALUX) reporter assay.

The CALUX panel consists of a range of assays, including those for major sex steroids (AR, ERα, ERβ, progesterone receptor CALUX assays), and other endocrine pathways (TRβ, glucocorticoid receptor, aryl hydrocarbon receptor CALUX assays etc.) (Piersma et al. 2013; Sonneveld et al. 2005; Van der Burg et al. 2013; Van der Linden et al. 2014). In 2015, the ERα CALUX bioassay was validated and approved according to the Organization for Economic Co-operation and Development (OECD) Test No.455: Performance-Based Test Guideline for stably transfected transactivation *in vitro* assays to detect estrogen receptor agonists and antagonists (Besselink 2015). Recently, the AR CALUX cell line was also validated and approved by the OECD: "Test no 458: Stably transfected human androgen receptor transcriptional activation assay for detection of androgenic agonist and antagonist activity of chemicals". The AR and ERα CALUX bioassays are particularly interesting for studying disruptions of the androgen-estrogen balance. Both bioassays were used in a pilot study involving ten breast milk samples, as detailed in Chapter Two. The AR CALUX bioassay was

further employed on 199 human milk samples, as we performed a case-study evaluating the possible association between anti-androgenic activity in breast milk and cryptorchidism occurrence in the offspring (Chapter Three).

During this PhD study, we also developed and validated CALUX reporters for THs, the TRβ CALUX, as well as a combination of TH plasma transport and TH receptor (TTR-TRβ-CALUX). For further description of these methods, we refer to Chapter Four of this thesis.

Effect-directed fractionation and identification of endocrine active compounds, including possible natural hormones

Bioassay-based analysis can provide excellent measurements regarding total hormonal activity in a sample, however, due to their structural similarities with natural hormones, EDCs are expected to be extracted along with endogenous compounds. Although natural hormones may play a critical role in EDC toxicity and therefore, cannot be excluded from the analysis, it is important to be able to discriminate the effects caused by EDCs actions from the endogenous nature hormone on CALUX-based measurements.

In order to isolate the active EDC fraction, a series of high-performance liquid chromatography (HPLC) fractionations was performed on a pooled breast milk sample. Following each separation, several extracts were collected and tested in a CALUX bioassay to identify the active fraction(s). The process of fractionation was continued until the active extract was pure enough to allow further chemical target analysis.

The purified active fraction was analyzed on HPLC high resolution quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF-MS/MS). A set of seven human natural steroid hormones (E1, E2, androstenedione, DHEA, testosterone, pregnenolone and progesterone) and 248 of their potential metabolites were selected for target analysis. The potential presence of these naturally occurring compounds in the active fraction was assessed and detailed in Chapter Two.

Scope of the thesis

The research described in this thesis investigated the amount and potential effects of EDCs present in human milk using samples derived from the Norwegian HUMIS cohort. This work focused on three main actors of the endocrine system, the estrogens, androgens, and thyroid hormones.

The first aim of the thesis was to develop a suitable method to properly extract EDCs from human milk samples, focusing on both polar and apolar endocrine active contaminants.

The second aim was to perform a pilot study on a limited number of breast milk samples, using the existing ERα and AR CALUX bioassays, in agonistic and antagonistic mode. This phase was also used to evaluate the nature and origin of the observed EDC activity, as well as the contribution of endogenous hormones to the measurements.

The third goal was to measure the EDC activity in a larger set of human milk samples to evaluate the potential association between anti-androgenic EDCs and an androgen-dependent deformity: cryptorchidism.

The last objective was the development and application of novel thyroid hormone-based bioassays, TRβ CALUX and TTR-TRβ CALUX assays. These bioassays were used to evaluate the impact of breast milk contaminants, including well-known PFAS (perfluorooctanoic acid or PFOA; perfluorooctane sulfonic acid or PFOS), on the thyroid system.

Outline of the thesis

The thesis is divided in two distinct parts: Chapter Two and Chapter Three focusing on sex steroid disruption, and Chapter Four and Chapter Five covering thyroid system interferences, respectively.

In Chapter Two, the method developed to extract EDCs from breast milk samples, used throughout the thesis, is described. In this chapter, estrogenic, anti-estrogenic, androgenic, and anti-androgenic activities derived from ten human milk extracts were analyzed on the (anti-)ERα and (anti-)AR CALUX bioassays. To rule out the impact of endogenous hormones in the measured activity, a pooled breast milk sample presenting anti-androgenic activity was fractionated and screened for natural hormones and metabolites by means of non-target screening using time-of-flight mass spectrometry UHPLC-Q-TOF-MS/MS.

Chapter Three describes a larger-scale study involving 199 participants and presents results of anti-androgenic EDC activity in breast milk samples. In this chapter, we investigated the potential association between anti-androgenic activity in mothers' milk and the occurrence of cryptorchidism, an androgen-dependent deformity, in the offspring. Moreover, we estimated the overall extent of exposure of a nursing child to anti-androgenic EDCs via breastfeeding.

In Chapter Four, the TRβ CALUX bioassay, allowing screening of TRβ disrupting activity, and the TTR-TRβ CALUX assay, designed to detect competing properties towards T4 for TTR binding, were evaluated using well-known reference compounds. The performance of the TTR-TRβ CALUX assay was also evaluated during a short pilot study involving water samples.

In Chapter Five the newly validated TRβ and TTR-TRβ CALUX bioassays were further used to assess a set of thirteen PFAS, known to affect the TH system (Chang et al. 2008; Thibodeaux et al. 2003) for thyroid-disrupting activities. Subsequently, ten breast milk extracts, with known PFOS and PFOA concentrations, were analyzed on the same assays. In this chapter, the impact of PFOS and PFOA levels was weighed in respect to thyroid-disrupting activity in breast milk. Furthermore, the average exposure to thyroid-disrupting EDCs during the first year of life of a nursing infant through breastfeeding was estimated.

Finally, Chapter Six reviews and discusses the most important outcomes of the studies regarding hormonal-disruption derived from the presence of EDCs in Norwegian breast milk samples. General conclusions and an outlook on future perspective are presented.

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Antagonistic Activity Towards the Androgen Receptor Independent from Natural Sex Hormones in Human Milk Samples from the Norwegian HUMIS Cohort

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Abstract

In this paper, we investigated the possible presence of endocrine disrupting chemicals (EDCs) based on measuring the total estrogenic and androgenic activity in human milk samples. We used specific bioassays for analysis of the endocrine activity of estrogens and estrogen-like EDCs and androgens and androgen-like EDCs and developed a separation method to evaluate the contribution from natural hormones in comparison to that of EDCs to total endocrine activities. We extracted ten random samples originating from the Norwegian HUMIS biobank of human milk and analyzed their agonistic or antagonistic activity using the ERα- and AR CALUX® bioassays. The study showed antagonistic activity towards the androgen receptor in 8 out of 10 of the assessed human milk samples, while 2 out of 10 samples showed agonistic activity for the ERα. Further investigations demonstrated anti-androgenic activity in the polar fraction of 9 out of 10 samples while no apolar extracts scored positive. The culprit chemicals causing the measured antagonistic activity in AR CALUX was investigated through liquid chromatography fractionation coupled to bioanalysis and non-target screening involving UHPLC-Q-TOF-MS/MS, using a pooled polar extract. The analysis revealed that the measured anti-androgenic biological activity could not be explained by the presence of endogenous hormones nor their metabolites. We have demonstrated that human milk of Norwegian mothers contained anti-androgenic activity which is most likely associated with the presence of anthropogenic polar EDCs without direct interferences from natural sex hormones. These findings warrant a larger scale investigation into endocrine biological activity in human milk, as well as exploring the chemical sources of the activity and their potential effects on health of the developing infant.

Introduction

Breast milk contains a complex mixture of proteins, lipids, carbohydrates as well as a high concentration of bioactive components, and is acknowledged to be important to the infant's post-natal growth and development. Beside its valuable properties, this biofluid also constitutes an important non-invasive source of information about the quality of the perinatal environment and its potential contamination (Esteban and Castaño 2009). Evaluation of toxicant levels in breast milk is an area of major interest, revealing the presence of diverse environmental contaminants such as persistent organic pollutants (POPs) and chemicals with estrogen-like properties (xenoestrogens) (Criswell et al. 2017; Massart et al. 2005; Thomsen et al. 2010). Xenoestrogens and several POPs are endocrine active and therefore referred to as endocrine active compounds (EACs). Usually at relatively high dosage levels, several EACs have been shown to lead to adverse effects, and these chemicals are referred to as endocrine disrupting chemicals (EDCs) (Bergman et al. 2012b). EACs and EDCs have been identified in a myriad of sources like contaminated food, indoor dust and daily-life products representing a challenge in their proper assessment and management. Their dose- and time-dependent effects can be particularly strong during vulnerable windows of development, from fetal life to the post-natal period up until puberty. Androgen- and estrogen signaling has been found to be a frequent target of hormonally active agents (Raun Andersen et al. 2002). While linked to adversities in wildlife and supported by circumstantial evidence, the linkage between early EDC exposures and human diseases is still not firmly established (Andersson et al. 2012; Colborn et al. 1993). Many different chemicals can interact with the estrogen- and androgen receptor and it has been shown that effects of EDCs can add up in mixtures (Rajapakse et al. 2002). Various epidemiological studies focused on investigating the possible relationship between early-life exposure to EDCs and child health outcomes, such as infant growth and impaired sexual development (Andersen et al. 2008; Iszatt et al. 2016a; Nørgaard et al. 2008). Exposure of Danish women workers to pesticides used in greenhouses has been associated with a rise in impaired reproductive development in their sons suggesting a link between EDC exposure and congenital deformity (Andersen et al. 2008). Although monitoring of selected chemicals provides precious information regarding biofluids' contamination, data considering the totality of these exogenous toxicants as well as the combined biological activities resulting from mixture effects are still limited.

Over the past decades, new biological, non-targeted testing procedures have been developed to supplement the targeted chemical-analytical techniques. Interestingly, it has been shown that single specific *in vitro* tests covering only a limited set of conserved pathways

can also provide very good predictions with respect to developmental and reproductive toxicity landmarks such as sex organ deformities (Van der Burg et al. 2014). These highly selective reporter gene methods to assess (anti-)estrogenic and androgenic activity, were also shown to be excellent alternatives to the traditional *in vivo* techniques in rodents and extremely suitable for measurements in complex mixtures such as body fluids (Pedersen et al. 2010; Sonneveld et al. 2006). Estrogen Receptor alpha (ERα) and Androgen Receptor (AR) mediated bioassays are based on two human U2-OS osteoblastic osteosarcoma cell lines stably transfected to endogenously express the ERα or AR, respectively. The activation of these specific receptors upon ligand stimulation triggers their binding to hormone-responsive elements (HRE), which is linked to a luciferase ("reporter") gene, leading to luciferase expression. By measuring the subsequent light production by luciferase, this bioassay can quantify hormonal activity of any chemical or sample. To ensure the robustness of the *in vitro* method, the ERα CALUX® bioassay was validated in 2015 according to the Organization for Economic Cooperation and Development (OECD) Test No.455: Performance-Based Test Guideline for stably transfected transactivation *in vitro* assays to detect estrogen receptor agonists and antagonists (Besselink 2015).

This study confirmed that the (anti-)ERα CALUX reporter gene bioassay is suitable for accurately predicting estrogen-disrupting activities. In turn, the (anti-)AR CALUX assay was recently implemented in the EU-NETVAL (European Union Network of Laboratories for the Validation of Alternative Methods) validation project, following the OECD Test Guideline Androgen Receptor Transactivation Assays and is currently in the process of an inter-laboratory evaluation. Over the years CALUX bioassays have been proven to be quick, specific methods able to measure the total effect of ligands on a receptor of interest using a limited volume of samples (Houtman et al. 2009; Kraus et al. 1995; Sonneveld et al. 2005).

Being the primary source of nutrition for most infants, breast milk contamination by exogenous chemicals could affect hormone-dependent mechanisms compromising post-natal growth. In that way, it is of major interest to assess the potential presence of EDCs in human milk as well as its short- and long-term impact on the developing child. In this paper we perform a pilot study aiming to extract EDCs from breast milk samples to further evaluate their impact on the endocrine system using the ERα and AR as endpoints. As a first step we demonstrate the performance of the AR CALUX bioassay, both in agonistic and antagonistic mode, by comparing our internal database to the *in vivo* Hershberger database recently established by Browne et al. (2018), as has been done to evaluate the ToxCast/Tox 21 AR model conducted by Kleinstreuer et al. (2017). As a second part, a set of ten breast milk samples derived from the "Norwegian Human Milk Study" (HUMIS), a birth cohort of mother-child

pairs, was analyzed. Due to the various biochemical properties of human milk contaminants, ensuring their total extraction from breast milk using one universal method appeared to be challenging. Therefore, we developed a two-step method capable of extracting apolar EDCs fraction and polar components, including endogenous hormones, separately. Both fractions along with a reconstituted mixture, consisting of the combination of both apolar and polar extracts, were analyzed following the workflow detailed in Fig.1. In addition to analysing the polar and apolar extracts, we also analysed a reconstituted mixture of the two fractions derived from each sample.

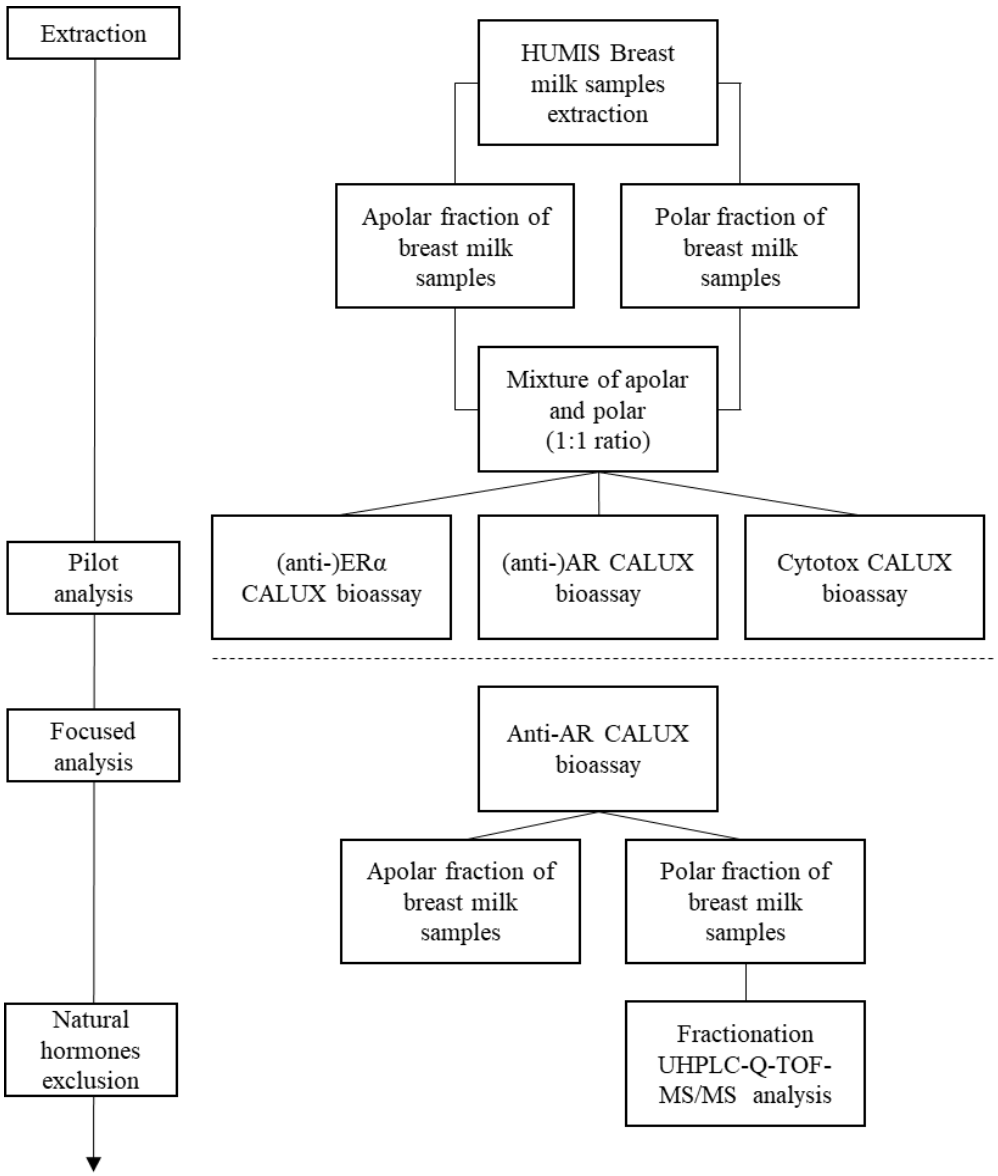

Figure 1. Study design of the extraction procedure and pilot analysis of ten breast milk samples on the (anti-)ERα and (anti-)AR CALUX bioassay

Materials & methods

Chemicals

17α-methyltestosterone (CAS: 58-18-4), 17β-estradiol (E2) (CAS: 50-28-2), 4-nonylphenol (CAS: 104-40-5), 5α-dihydrotestosterone (DHT) (CAS: 521-18-6), amitrol (CAS: 69182-5), bis-(2-ethylhexyl)phthalate (DEHP) (CAS: 117-81-7), bisphenol A (BPA) (CAS: 80-05-7), chlorothalonil (CAS: 1897-45-6), chlorpyrifos (CAS: 2921-88-2), dibutyl-phthalate (DBP) (CAS: 84-74-2), estrone (E1) (CAS: 53-16-7), fenarimol (CAS: 60168-88-9), flutamide (CAS: 13311-84-7), flutolanil (CAS: 66332-96-5), glyphosate (CAS: 1071-83-6), linuron (CAS: 330-55-2), *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE) (CAS: 72-55-9), pregnenolone (CAS: 145-13-1), procymidone (CAS: 32809-16-8), progesterone (CAS: 57-83-0), tamoxifen (CAS: 10540-29-1), testosterone (CAS: 58-22-0) and vinclozolin (CAS: 50471-44-8) were obtained from Sigma-Aldrich (The Netherlands). 4-androstan-17b-ol-3-one (androstenedione) (CAS: 63-05-8) and 5-androstan-3b-ol-17-one (DHEA) (CAS: 53-43-0) were purchased from Steraloids Inc. (USA). Tributyltin acetate (CAS: 56-36-0) was obtained from Merck Chemicals B.V. (The Netherlands).

Cell lines

The AR CALUX, ERα CALUX and Cytotox CALUX bioassays (Sonneveld et al. 2005; Van der Linden et al. 2014) are based on a stably transfected human osteoblastic osteosarcoma U2-OS cell-line (American Type Culture Collection). The highly selective cell line used in the (anti-)AR CALUX contains a full-length human AR expression vector stably co-transfected with a reporter construct containing a minimal promoter element, the TATA box, coupled to a luciferase reporter construct containing three androgen responsive elements (Sonneveld et al. 2005). The (anti-)ERα CALUX contains a similar expression vector expressing human ERα and a reporter construct 3xpERE-TATA-Luc, as described earlier (Sonneveld et al. 2005). Both cell lines were also used to perform the antagonistic (anti-) AR and ERα CALUX bioassays. As a control to detect non-specific activities (e.g. cellular death), each antagonistic measurement was performed along with the Cytotox CALUX, consisting of U2-OS cells constitutively expressing the luciferase gene (Van der Linden et al. 2014). The threshold was set at cytotoxicity ≤ 20%. For evaluation of analysis data, concentrations surpassing this value were excluded. All CALUX cell lines were cultured as described previously (Sonneveld et al. 2005).

Reference compound data selection

The Hershberger *in vivo* androgenicity assay database established by Browne et al. (2018) was browsed to establish a set of reference chemicals for the evaluation of the performance of the (anti)AR CALUX. Chemicals with consistent effects in Hershberger study were pre-selected. Compounds present in both *in vivo* database and internal CALUX dataset were chosen for the final list. A total of sixteen reference compounds including six non-active chemicals (4-nonylphenol, amitrol, chlorothalonil, chlorpyrifos, flutolanil and glyphosate), eight AR antagonists (DBP, DEHP, fenarimol, flutamide, linuron p,p'-DDE, procymidone and vinclozolin) and two steroidal androgens (17α-methyltestosterone and testosterone) were selected on that basis. The internal (anti-)AR CALUX database gathers more than 200 pure compounds individually analyzed using an automated version of the (anti-)AR CALUX.

Human milk samples

Samples were derived from the mother-child cohort study HUMIS (Human Milk Study), cooperatively conducted by the Norwegian Institute of Public Health between 2002 and 2009 (Eggesbø et al. 2004). Between the first two weeks and months after delivery, women enrolled in the study were asked to collect 25 mL of breast milk every morning for eight consecutive days, preferably by hand. Milk aliquots were collected in 250 mL natural HDPE Packaging Bottles (Cat. No.: 967-21244, Thermo Scientific Nalgene®) made from high-purity resins, a food-grade material free of plasticizers. Aliquots were sent by the mothers along with a questionnaire gathering the following information: maternal age, weight, height, residence, smoking habits, parity, nationality, education, work and dietary habits. Samples were stored upon arrival at -20 °C in a Biobank of the Norwegian Institute of Public Health. The study was approved by the Norwegian Data Inspectorate (ref. 2002/1398) and Regional Ethics Committee for Medical Research (ref. S-02122). Mothers were included after oral and written informed consent had been obtained.

Sample preparation

For this study, ten aliquots were used to extract apolar and polar components, following the methods described below.

For apolar compounds, 5 mL of homogenized milk sample was transferred to a 60 ml glass tube. The same amount of 2-propanol (CAS: 67-63-0, BioSolve) was added prior to the extraction to optimize the penetration of n-hexane into the sample material during the next stage. Tubes were shaken for 10 min on a shaker at 200±20 strokes per minute. 14 mL of n-

hexane (CAS: 110-54-3, BioSolve) was added and the tubes were shaken for an extra hour. The upper layers were transferred to clean collection tube and the procedure was repeated twice with a shaking time reduced to 30 min. The collected fractions were evaporated to dryness and reconstituted in 1 mL of n-hexane. The extracts were cleaned (including fat removal) using glass columns filled with 5 g of 2% deactivated silica, previously conditioned with 12 mL of n-hexane. The samples were eluted with 30 mL of a 3:1 n-hexane and dichloromethane mixture (CAS: 75-09-2, BioSolve). The eluate obtained was evaporated to dryness and reconstituted in 30 µL of DMSO.

The efficiency of the apolar extraction was assessed by including four procedural controls extracted along the set of breast milk samples, using gas chromatograph coupled with tandem mass spectrometer (GC-MS/MS) as the detection technique. The controls consisted of 5 mL of a pooled breast milk sample spiked with 50 µL of ^{13}C-labeled internal standard solution containing PCB153 (2,2',4,4',5,5'-hexachlorobiphenyl) and PCB180 (2,2',3,4,4',5,5'-heptachlorobiphenyl) (200 ng/mL, diluted from MBP-D7, Wellington Laboratories). Due to their apolar properties and their known presence in breast milk, PCB153 and PCB180 were suitable contaminants to evaluate the performance of the extraction method (IARC Working Group on the Evaluation of Carcinogenic Risk to Humans. 2016). Controls were extracted following the same procedure as used for samples with the exception that they were dissolved in 1 mL of isooctane (CAS: 540-84-1, BioSolve), which is a more suitable and common solvent for chemical analysis with GC-MS/MS. A recovery standard containing PCB112 (10 ng/mL, diluted from C-112S-TP, AccuStandard) was added to each control extract just prior to the injection. This extra step was included to correct for response variations due to internal matrix effects during GC-MS/MS measurement, and is independent from the extraction performance. Internal standard recoveries were evaluated on a GC-MS/MS system, consisting of a gas chromatograph (GC-2010 Plus, Shimadzu) and a gas chromatograph mass detector (GCMS-TQ8050, Shimadzu), both controlled by the GCMS Real Time Analysis software program (available at: https://www.ssi.shimadzu.com/products/gas-chromatography-mass-spectrometry/gcmssolution-software.html). The injection was carried out using a CTC CombiPal autosampler controlled by the Cycle Composer software program (CTC Analytics AG). The system equipped with a DB-5MS column (60m x 0.25mm x 0.25µm) (Cat. No.: 122-5562, Agilent Technologies), operated using the following parameters: carrier gas Helium 6.0 (BIPX10S, Air Products), constant flow rate of 1.00 mL/min (electronically controlled), injection temperature 280.0 °C, injection volume 1 µL, splitless mode, ion source temperature 230.0 °C, interface temperature 300.0 °C. The analysis was performed using the parameters and settings described in Tables 1,2. After PCB112 correction, recovery values were evaluated to 92±5.4 and 91±4.1% for PCB153 and PCB180, respectively.

Table 1. GCMS oven temperature program for recovery assessment.

Rate	Temperature (°C)	Hold Time (min)
-	80.0	2.0
20.00	180.0	0.0
5.00	200.0	0.0
2.00	240.0	10.0
20.00	320.0	10.0

Table 2. GCMS internal and recovery standards for recovery assessment.

Internal Standard	Precursor	Product
PCB153L (m/z)	371.80	301.90
PCB180L (m/z)	405.80	335.90

Recovery Standard	Precursor	Product
PCB112 (m/z)	323.90	253.90

Note: m/z: mass-to-charge ratio.

QuEChERS (Quick Easy Cheap Effective Rugged and Safe) was used to extract polar compounds. It is a simplified sample extraction technique developed to assess multiple pesticide residues in food (Anastassiades et al. 2003). We used a protocol derived and adapted from the original method, as described below. Samples were homogenized and 5 mL was transferred to a 50 mL Greiner tube. 15 mL of acetonitrile (ACN) (CAS: 75-05-8, BioSolve) was added as the extraction solvent and the mixture was shaken vigorously manually for 30 seconds. One QuEChERS EN 15662 extraction packet (Cat. No.: 5982-5650, Agilent) containing 4 g of magnesium sulfate, 1 g of sodium chloride, 1 g of sodium citrate dehydrate and 0.5 g of sodium hydrogen citrate sesquihydrate was added directly into the tube and shaken strongly for 15 min. The tubes were centrifuged for 5 min at 4000 rpm at 4 °C and the upper layers were transfer into a clean collection tube. The same procedure, including ACN and addition of salts, was repeated once and the resulting layers were combined. Each combined extract was loaded to a 15 mL QuEChERS dispersive solid phase extraction (d-SPE) column (Cat. No.: 5982-5158, Agilent) for clean-up and vortexed for 1 min. The tubes were centrifuged for 5 min at 4000 rpm at 4 °C. The upper layer was transferred to a clean collecting

tube and evaporate until dryness. The samples were reconstituted in 30 µL of DMSO and stored at -20 °C until analysis.

Reproducibility and efficiency of the polar extraction was assessed by adding four controls to the analysis series. The controls consisted of 5 mL of breast milk spiked with 100 µL of a mixture of BPA, E2 and testosterone as internal standards (100 µg of each compound/mL). Due to their polarity and their known presence in breast milk, BPA (logP = 4.0), E2 (logP = 3.7) and testosterone (logP = 3.4) were three appropriate compounds to assess the efficiency of the polar extraction method (Mendonca et al. 2014). Controls and samples were processed following the same extraction procedure, except that controls were redissolved in 30 µL ACN, a suitable and common solvent for the liquid chromatography (LC) analysis of these components. Chemical analysis was performed by LC using a modular system from Agilent consisting of 1260 Infinity High Performance Degasser (G4225-64000), a 1260 Infinity Binary pump (specified up to 600 bar - G1312B), Multisampler (G7167-64000, Agilent), Diode array detector (G1315b-64050, Agilent) and a thermostatted column compartment (G1316-64050, Agilent). The samples were analyzed on a Phenomenex Kinetex Biphenyl column (150x4.6mm 2.6µ particle size) (00F-4622-E0, Phenomenex) using the following parameters: injection volume 20 µL; flow rate of 0.8 mL/min; detection (UV) 254, 272 nm; column temperature 40 °C. The system was controlled by Agilent OpenLAB CDS (EZ ChromEdition) software program. This program was also used to process the data. Water and ACN were used as solvents according to the scheme described in Table 3. Recovery values were calculated by means of comparison of the peak height in the control samples with a standard solution. BPA, E2 and testosterone were recovered to a rate of 36±13, 50±7.1 and 61±2.9 %, respectively. On average, 49% of spiked polar chemicals were recovered after extraction. This result was used to compensate for loss of signal during the CALUX analysis, i.e. polar measurements were adjusted to apolar results using a factor of 2. Moreover, polar and apolar ratio to reconstitute the mixture (originally 1:1) was adapted to insure the correctness of the study (2:1). All extracts were kept at -20 °C until analysis.

Table 3. Liquid chromatography gradient of water/acetonitrile for recovery assessment.

Time (min)	Water (%)	ACN (%)
0.00	30.0	70.0
1.00	30.0	70.0
10.00	0.0	100.0
12.00	0.0	100.0
12.50	50.0	50.0
17.50	50.0	50.0

Note: ACN: Acetonitrile.

CALUX assay procedures

Human milk samples were extracted, and bioactivities were evaluated with the (anti-)ERα and (anti-)AR reporter gene CALUX (Chemically Activated Luciferase eXpression) bioassays at different concentrations (dilution series 1-3-10-30-100x). The (anti-)ERα and (anti-)AR CALUX bioassays were carried out typically as described by Sonneveld et al. (2005). In short, cultured U2-OS cells were re-suspended in DMEM/F12 medium without phenol red indicator (Fisher, product no.: VX1041025) with 10 U/mL penicillin and 10 µg/mL streptomycin (P/S), non-essential amino acids (NEAA) (Cat No.: 11140-03, Gibco) and 5% charcoal-stripped fetal calf serum (DCC) supplemented. Cells were re-suspended in assay medium to a final concentration of $1x10^5$ cells/mL, subsequently 100 µl were seeded in clear 96-well plates. Plates were incubated for a minimum of 16 hours and maintained in a humidified atmosphere at 37 °C and 5% CO_2. After a maximum of 24 hours, the medium was replaced with 200 µL/well of medium including the sample to a final DMSO concentration of 0.1%. To perform the antagonistic mode, the medium was supplemented with a fixed concentration of E2 (EC50: $6.0E^{-12}$ M) or dihydrotestosterone (DHT; EC50: $3.0E^{-10}$ M), for the anti-ERα and anti-AR CALUX, respectively. After 22±2-hour incubation, the medium was removed and 30 µL/well of Triton-lysis buffer was added and luciferase activity was measured using a Tristar luminometer (Berthold).

Analysis of pure compounds using the AR CALUX was essentially performed as above with the exception that the dilution (0.5 log unit increments) and the exposure was automated and performed in a 384-well plate according to a validated method as to increase the efficiency of the procedure (Van Vugt-Lussenburg et al. 2018).

All dilutions including the ones from the samples were analyzed in triplicate on the same 96-well plate. A solvent control (DMSO) was included to each plate to assess the background luciferase activity. Moreover, a full dose response curve of the reference compound E2 (ERα CALUX), DHT (AR CALUX) or tributyltin acetate (Cytotox CALUX) was included to each plate. The reference compounds tamoxifen and flutamide were used as reference for the anti-ERα and anti-AR CALUX assays, respectively. Antagonistic measurements were double-checked for non-specific interactions by assessing test compounds with a saturating excess amount of agonist i.e. 1000-times the EC50 concentration of the agonistic reference compound (Van der Burg et al. 2010). The quality and validity of the measurements were evaluated according to the following criteria: R2 of standard curve > 0.98, EC50 of the reference compound should fit between assay-specific predetermined limit values, z-factor of standard curve > 0.6 and overall SD of triplicate < 15%.

Data analysis

Luciferase activity was expressed as Relative Light Units (RLUs). The average of each triplicate was calculated and corrected for background using solvent control measurements. The maximum signal response induced by the reference compound was set to 100% for agonistic assay (complete activation of the receptors) and 0% for antagonistic mode (complete inhibition of the signal). Subsequently, all RLUs produced by the test compounds or samples were expressed as a % of this maximum response. The statistical software package GraphPad Prism 5.0 was used to fit transformed data (non-linear regression, variable slope, four parameters and robust fit). The lowest observed effect concentrations (LOECs) were determined; for agonist assays, the LOEC was defined as the PC10 value, while for antagonist assays a PC20 value was used. The PC10 concentration was defined as the concentration where the response elicited by the test substance equals 10% of the maximum response of the reference substance. PC20 values were defined as the concentration where the test substance causes 20% inhibition of the basal signal elicited by the receptor agonist. In this case, the maximum inhibition achieved by the reference antagonist was set at 100%. Compounds without a calculable PC10/PC20 value were defined as non-active (NA). For quantification of the (anti-)estrogenic and (anti-)androgenic potency of breast milk extracts, RLUs derived from CALUX analysis were plotted to the dose-response curve of the associated reference compound. A sample was reported as ‘active’ if its overall activity was above the limit of quantification (LOQ) value of the bioassay, i.e. lowest concentration that can be quantified with acceptable relative standard deviation. A non-active sample i.e. activity ≤ LOQ, was replaced by half of the LOQ value to give an estimation of the activity. The calculated

concentrations per well (M) were converted and expressed as equivalence of the reference compound per gram of processed sample.

Instrumentation and analytical methods for fractionation

Seven natural steroid hormones, E1, E2, testosterone and their precursor androstenedione plus pregnenolone, progesterone and their product DHEA and androstenedione were targeted during an exclusion by fractionation using liquid chromatography. Androstenedione, DHEA and testosterone were selected based on their androgenic properties that could create interferences with AR, possibly counteracting anti-AR activity measured in the CALUX bioassay (Burger 2002; Houtman et al. 2009). On the other hand, E1, E2, progesterone and pregnenolone were chosen for their estrogenic characteristics. This type of activity was earlier reported to be anti-androgenic and androgenic, depending on the concentration, which could also bias the measurements (Kelce and Wilson 1997; Sohoni and Sumpter 1998; Van der Burg et al. 2010).

The outlet of the high-performance liquid chromatography (HPLC) setup described earlier was connected to a Fraction collector III (WFCIII, Waters) remotely triggered by the Agilent system. In the fractionation process we used two types of columns, Phenomenex Kinetex Biphenyl 150x4.6mm 2.6u particle size (00F-4622-E0, Phenomenex) and Phenomenex Kinetex Fluor 5 100x2.1mm 1.7u particle size (00D-4722-AN, Phenomenex) (pentafluorophenyl stationary phase bonded to a solid silica core). We operated the described system with a combination of methanol absolute HPLC Supra-gradient (CAS: 67-56-1, BioSolve) and ultra clean water from an ELGA filtration station as solvent. For all experiments the columns were maintained at 40 °C. Data were analyzed using the Agilent OpenLAB CDS (EZ ChromEdition) software program. Parameters including column, solvents and gradients used for each fractionation are referred to as ‘protocol n°1’ and ‘protocol n°2’ (Table 4.).

Table 4. Liquid chromatography conditions, column and gradient used for fractionation.

	Column	Time (min)	Water (%)	MeOH (%)
Protocol n°1	Phenomenex Kinetex Biphenyl	0.00	50.0	50.0
	150x4.6mm 2.6u particle size	0.01	50.0	50.0
	0.8mL/min	10.00	5.0	95.0
	Injection vol. 30µL	16.00	5.0	95.0
		16.50	50.0	50.0
		25.00	50.0	50.0
Protocol n°2	Phenomenex Kinetex Fluor 5	0.00	80.0	20.0
	100x2.1mm 1.7u particle size	0.01	80.0	20.0
	0.250mL/min	25.00	0.0	100.0
	Injection vol. 20 µL	28.00	0.0	100.0
		28.50	80.0	20.0
		48.00	80.0	20.0

Instrumental settings for chemical analysis followed by non-target screening

The active fractions along with non-active ones, collected adjacent to the active eluates, were obtained through fractionation and were analyzed in triplicate with an Nexera UHPLC system (Shimadzu, Den Bosch, the Netherlands), coupled to a maXis 4G high resolution quadrupole time-of-flight HRMS (UHPLC-Q-TOF-MS/MS) upgraded with a HD collision cell and equipped with an electrospray ionisation source (ESI) (Bruker, Leiderdorp, the Netherlands) (Appendix A.1). Liquid chromatography analysis was performed according to protocol n°2, described earlier (Table 4). 2 mM of sodium formate in 1:1 water/MeOH solution was used for automatic internal calibration of the system. Positive and negative ESI was used to acquire MS- and MS/MS data in the range of 80 - 1200 m/z. Simultaneously an UV chromatogram (254 nm) was acquired using an UV detector placed in line between the UHPLC and the Q-TOF-MS/MS. The peak retention times in the UV chromatogram were compared to those found during the fractionation to compensate for differences between both LC systems.

The data acquired from the non-target analysis (NTA) was processed using an in-house developed R package 'patRoon' (Helmus et al. 2018) which is an open-source platform that

harmonizes various commonly used software tools employed in NTA. More information is available at *https://github.com/rickhelmus/patRoon (Patroon: Open Source Software Platform for Environmental Mass Spectrometry Based Non-target Screening 2020)*. The first step of the patRoon workflow consisted of converting the manufacturer specific data to open mzML format. Second, features (single peaks within the extracted ion chromatogram) were extracted from all analysed fractions and grouped according to retention time and m/z (mass to charge ratio). Third, the features were filtered by employing different constraints and thresholds. The feature data were filtered following these steps:

- Removing any features below an intensity threshold (10000).
- Removing features that are not at least 5 times higher in intensity compared to the blank.
- Remove features that are not present in all triplicate injections and with intensity variations amongst triplicates of >75% RSD.
- Only unique features that are present in active fractions were kept, features from non-active fractions were removed.

Next, possible human metabolites of the seven endogenous hormones were predicted using BioTransformer (*http://biotransformer.ca/*) and the software R. Based on all known reaction pathways from BioTransformer, a list of 248 metabolites was generated (Appendix A.1). The presence of these metabolites in the active fractions was determined by comparing their m/z (within a window of 0.002 Da) to those of the unique post-processed features for the background.

Results

Evaluation of the CALUX assay performance

In order to test the performance of the (anti-)AR CALUX assays relative to reference methods we used a similar approach as has been used for the ERα CALUX assay (Vugt-Lussenburg et al. 2018). A set of sixteen reference compounds including six non-active chemicals, eight AR antagonists and two steroidal androgens were selected based on the Hershberger *in vivo* androgenicity assay database developed by Browne et al. (2018) (Table 5). In the agonistic mode, only two steroids, 17α-methyl testosterone and testosterone, had sufficient reference data. They were evaluated and found to be strong agonists with a PC10 of -9.6. This is consistent with earlier findings that this assay can predict androgenicity of chemicals, including steroids, and is predictive for androgenicity as determined in the Hershberger assay (Houtman et al. 2009; Sonneveld et al. 2006). Expectedly, none of these agonists were active in the anti-AR CALUX assay. A larger set of eight reference anti-androgens with consistent effects in the *in vivo* Hershberger assay was assessed in the anti-AR CALUX assay. All chemicals showed anti-androgenic activity, with the exception of DEHP which showed no response at the highest tested concentration (-4). This is consistent with the fact that DEHP is not a receptor antagonist but rather decreases circulating levels of androgens (Borch et al. 2006). Six chemicals (4-nonyphenol, amitrol, chlorothalonil, chlorpyrifos, flutolanil, glyphosate), which demonstrated no effect in the *in vivo* Hershberger model, scored negative in both the agonist and antagonistic modes of the AR CALUX assay. Overall, the AR CALUX assay demonstrated a very satisfying predictivity suggesting that this assay is suitable to assess androgen receptor-mediated effects of chemicals.

Table 5. Summary of the comparison between (anti-)AR CALUX results and in vivo reference classification.

CAS no.	Compound	*in vivo* Reference Classification	AR CALUX PC10* (logM)	Anti-AR CALUX PC20* (log M)
58-18-4	17α-methyl testosterone	androgenic	-9.6	NA (>-4)
58-22-0	testosterone	androgenic	-9.6	NA (>-4)
84-74-2	DBP	anti-androgenic	NA (>-4)	-4.7
117-81-7	DEHP	anti-androgenic	NA (>-4)	NA (>-4)
60168-88-9	fenarimol	anti-androgenic	NA (>-4)	-6
13311-84-7	flutamide	anti-androgenic	NA (>-4)	-6.5
330-55-2	linuron	anti-androgenic	NA (>-4)	-6.3
72-55-9	*p,p'*-DDE	anti-androgenic	NA (>-4)	-7.5
32809-16-8	procymidone	anti-androgenic	NA (>-4.5)	-7.3
50471-44-8	vinclozolin	anti-androgenic	NA (>-4)	-6.7
104-40-5	4-nonylphenol	negative	NA (>-4)	NA (>-4)
61-82-5	amitrol	negative	NA (>-4)	NA (>-4)
1897-45-6	chlorothalonil	negative	NA (>-4)	NA (>-4)
2921-88-2	chlorpyrifos	negative	NA (>-6)	NA (>-6)
66332-96-5	flutolanil	negative	NA (>-4)	NA (>-4)
1071-83-6	glyphosate	negative	NA (>-4.3)	NA (>-4.3)

Note: *PC10 (agonistic mode) or PC20 (antagonistic mode), i.e., the concentration where the response elicited by the test compound equals 10 or 20% of the maximum response were calculated. Abbreviations: DHT: 5α-dihydrotestosterone; DEHP: bis-(2-ethylhexyl)phthalate (DEHP) (CAS: 117-81-7); *p,p'*-DDE: *p,p'*-dichlorodiphenyldichloroethylene. NA: no activity observed at the highest concentration tested; value in between brackets is the highest concentration tested.

Determination of hormone-related activity in breast milk samples

Next, we used the AR CALUX along with the ERα CALUX assay to assess the hormonal activity in ten breast milk samples. To optimally extract chemicals from the breast milk, apolar and polar analytes derived from each sample were extracted using two separate methods and combined subsequently to form reconstituted extracts. These mixtures were analyzed in the ERα and AR CALUX bioassays, both in the agonistic- and antagonistic mode (Table 6). All samples were first tested and corrected for cytotoxicity to exclude non-specific effects. No antagonistic activity towards ERα was observed and only two samples scored positive in the agonistic ERα CALUX bioassay. All samples demonstrated anti-AR activity except number 1 and 8 which did not reach the limit of quantification (LOQ) of the AR CALUX bioassay.

Considering that 80% of breast milk samples were positive for anti-androgenic testing, a migration experiment was performed according to European Commission Regulation No 10/2011 and EN1186, described by Kirchnawy et al. (2014) to assess possible artificial activities derived from plastic HDPE bottles. Results demonstrated that an anti-androgenic activity equals to 0.18±0.04 μg flutamide eq./g of material could be attributed to the containers. These findings showed that only a small proportion of the activity present in human milk samples could be explained by storage conditions, however all active samples were well above this value suggesting that they contain biologically active components with anti-androgenic activity. As a first step towards understanding the nature of the anti-AR activities, the same ten human milk samples were analyzed as two distinct fractions.

In all apolar fractions the antagonistic activities detected were below LOQ (Table 7). All polar fractions scored positive ranging from 1.8 to 9.0 μg flutamide eq./g with the exception of sample n°1 which was classified as <LOQ due to its extremely low signal. Anti-androgenic activity in polar fractions were found to be significantly higher than the average background levels from leachable components. All activities measured in the polar extracts were considerably higher when assessed alone rather than in combination with the apolar fraction as a reconstituted mixture. These results suggest an accumulation of polar compounds, possibly EACs with AR antagonistic properties in breast milk of the HUMIS cohort. However further investigation is required to determine whether the anti-AR activity is caused by endogenous compounds in human milk i.e., natural hormones, or is of anthropogenic nature.

Table 6. Hormonal activity of ten reconstituted human milk samples assessed in the (anti-)AR and (anti-)ERα CALUX bioassays.

Number	ERα CALUX (ng E2 eq./g)	Anti-ERα CALUX (µg tamoxifen eq./g)	AR CALUX (ng DHT eq./g)	Anti-AR CALUX (µg flutamide eq./g)
1	0.15	0.15 (<LOQ)	4.0	0.22 (<LOQ)
2	0.002 (<LOQ)	0.15 (<LOQ)	0.02 (<LOQ)	0.73
3	0.002 (<LOQ)	0.15 (<LOQ)	0.02 (<LOQ)	0.53
4	0.002 (<LOQ)	0.15 (<LOQ)	0.02 (<LOQ)	0.58
5	0.14	0.15 (<LOQ)	0.02 (<LOQ)	0.61
6	0.002 (<LOQ)	0.15 (<LOQ)	0.02 (<LOQ)	0.85
7	0.002 (<LOQ)	0.15 (<LOQ)	0.02 (<LOQ)	0.64
8	0.002 (<LOQ)	0.15 (<LOQ)	0.02 (<LOQ)	0.23 (<LOQ)
9	0.002 (<LOQ)	0.15 (<LOQ)	0.02 (<LOQ)	0.61
10	0.002 (<LOQ)	0.15 (<LOQ)	0.02 (<LOQ)	0.8

Note: Each human milk sample was individually reconstituted from apolar and polar extracts. Samples are arbitrarily numbered from 1 to 10. Results are expressed as equivalent per gram of material using the reference compound of the assay as reference. Abbreviations: AR: androgen receptor; ERα: estrogen receptor α; E2: 17β-estradiol; DHT: 5α-dihydrotestosterone; LOQ: Limit of quantification.

Table 7. AR antagonistic activities of breast milk sample extracts measured using the antagonistic mode of the AR CALUX bioassay.

Number	Apolar (µg flutamide eq./g)	Polar (µg flutamide eq./g)	Combined extract (µg flutamide eq./g)
1	0.22 (<LOQ)	0.05 (<LOQ)	0.22 (<LOQ)
2	0.15 (<LOQ)	2.82	0.73
3	0.13 (<LOQ)	1.8	0.53
4	0.18 (<LOQ)	3.0	0.58
5	0.2 (<LOQ)	9.0	0.61
6	0.22 (<LOQ)	4.8	0.85
7	0.22 (<LOQ)	5.7	0.64
8	0.23 (<LOQ)	6.3	0.23 (<LOQ)
9	0.17 (<LOQ)	1.9	0.61
10	0.22 (<LOQ)	4.2	0.8
Average	<LOQ	4.0	0.58

Note: Apolar and polar endocrine active compounds derived from nine human milk sample were extracted and analyzed as two distinct fractions on the anti-AR CALUX. Both extracts were mixed and analyzed together to evaluate the effects of the combined fractions. Results are expressed as flutamide equivalent per gram of milk. Abbreviations: AR: androgen receptor; LOQ: Limit of quantification.

Chemical evaluation of the anti-androgenic activity in human milk samples

Human naturally occurring steroid hormones (e.g. DHEA, E2, E1, pregnenolone, progesterone and testosterone) could potentially interact with the androgen receptor. Using a pooled breast milk sample as a basis, we evaluated if this set of hormones is related to the AR antagonistic activity as measured in the polar fraction. For this, we used effect-directed analysis to see if the biological effect (AR antagonism) coelutes with these steroids using a chromatographic separation step based on a Biphenyl column. Four distinct fractions, A.1 to A.4, were collected using HPLC following a five-minute time-interval scheme (Figure 2). Clearly, the steroids co-eluted with the bioactivity as an activity of 0.88 µg flutamide eq./g was measured in fraction number 3 (A.3) while no activity was present in the other fractions. Further fractionation was required to eliminate them from the extract.

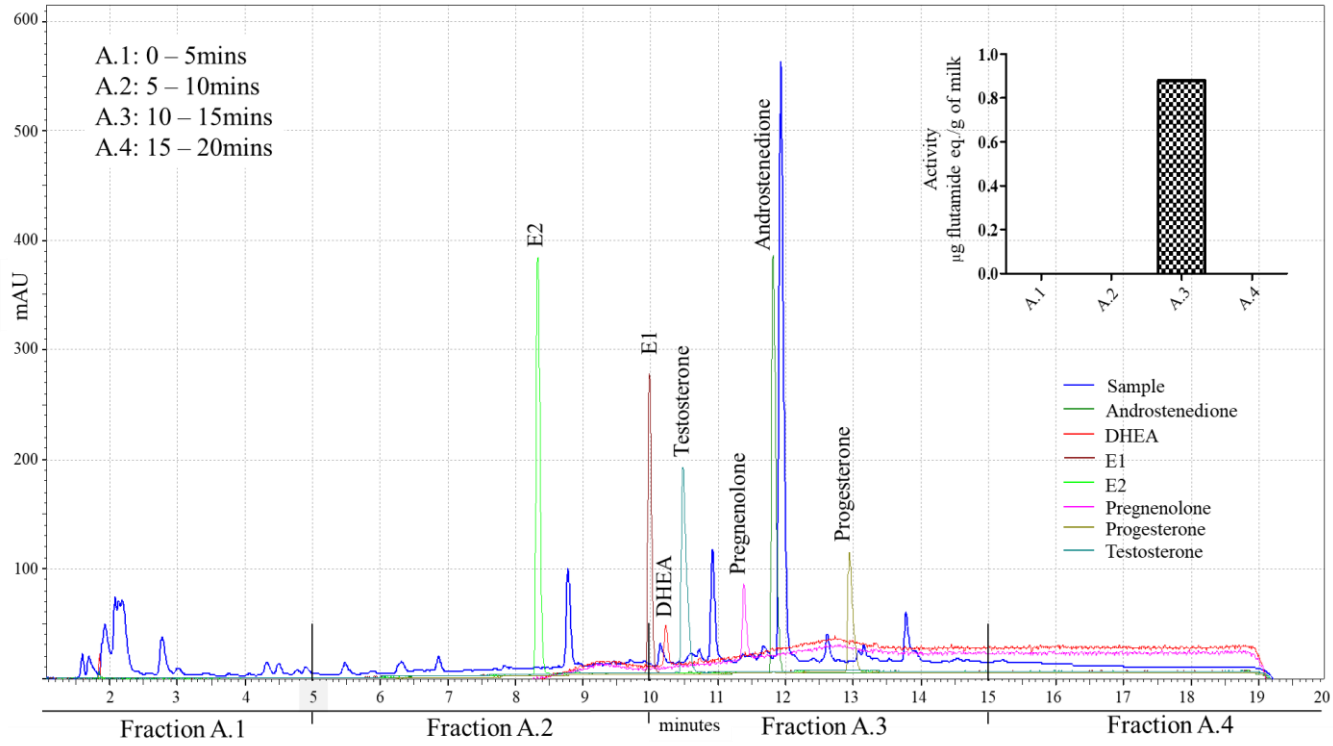

Figure 2. Chromatographic overlay of elution patterns of the seven endogenous hormones with the pooled human milk extract.

Note: Chromatogram derived from each individual internal standard was overlapped to the chromatogram of the polar fraction of a pooled breast milk sample (blue). All endogenous hormones except for E2 co-eluted with fraction A.3. Abbreviations: E1: estrone; E2: 17β-estradiol; DHEA: 5-androstan-3b-ol-17-one.

A.3 was further fractionated using a Fluor 5 column. With the exception of pregnenolone and progesterone, all the hormones co-eluted within fraction B.2, while only fraction B.3 showed an AR antagonistic activity (1.7 µg flutamide eq./g) (Figure 3). Due to their co-elution with the active fraction B.3, pregnenolone and progesterone could still play a role in the observed anti-androgenic activity in human milk samples.

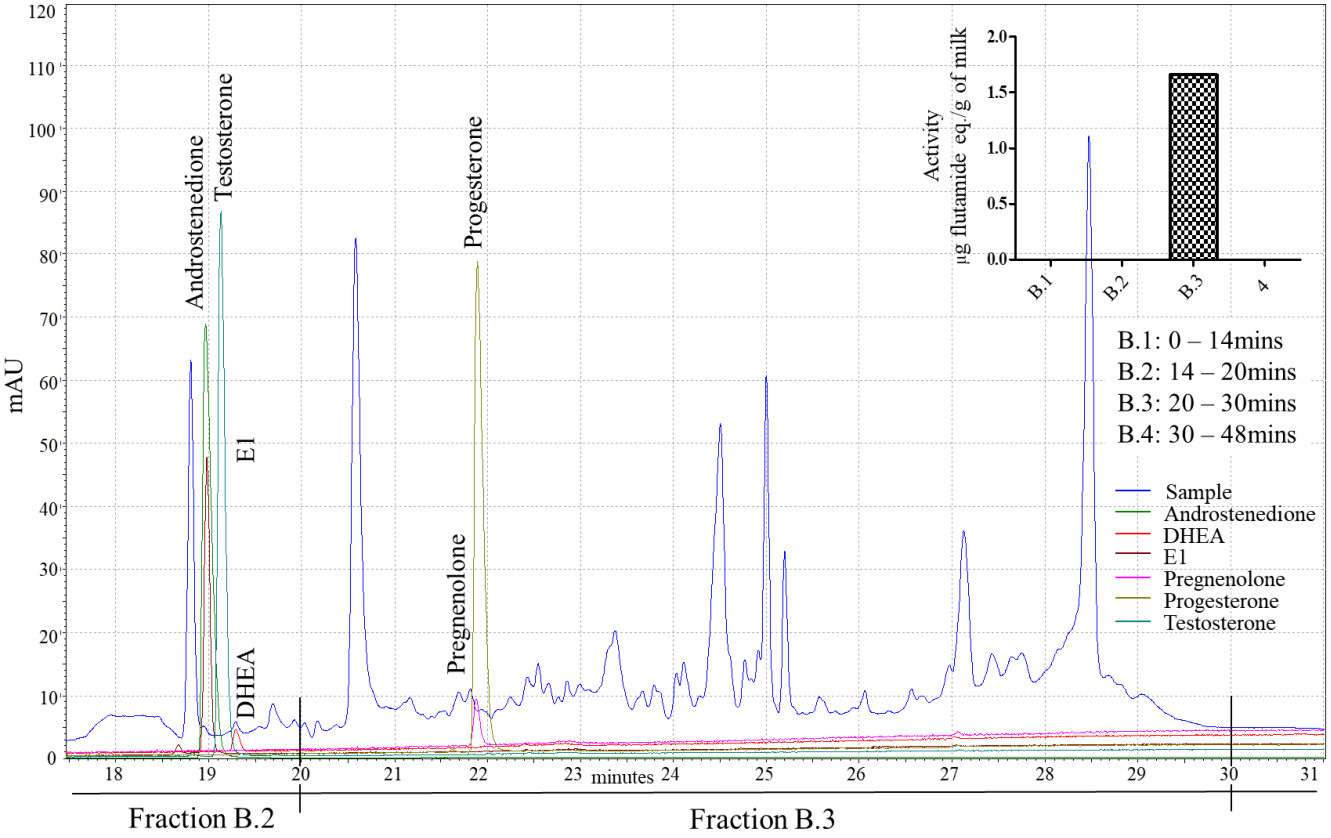

Figure 3. Chromatographic overlay of elution profiles of endogenous hormones and anti-AR bioactivity in human milk fraction A.3 on a Phenomenex Kinetex Fluor 5 column.

Note: Chromatogram derived from each individual internal standard was overlapped to the chromatogram of the polar fraction of a pooled breast milk sample (blue). All endogenous hormones except for pregnenolone and progesterone (b.3) co-eluted with fraction B.2. Abbreviations: E1: estrone; DHEA: 5-androstan-3b-ol-17-one.

The Fluor 5 chromatographic separation procedure was repeated, using smaller time window collection points. As the anti-AR activity was positioned in fraction B.3 (20 to 30 min), this 20-30 min time frame was further divided in five two-minute fractions, labelled C.1 to C.5, which were collected and analyzed separately in the anti-AR CALUX (Figure 4). Fractions C.3 and C.4 were found to contain anti-androgenic activity with a total activity of the combined fractions equivalent to 1.1 µg flutamide eq./g. Pregnenolone and progesterone co-eluted with fractions C.1 and C.2 implying that none of the studied endogenous hormones were in the extract containing the anti-AR activity.

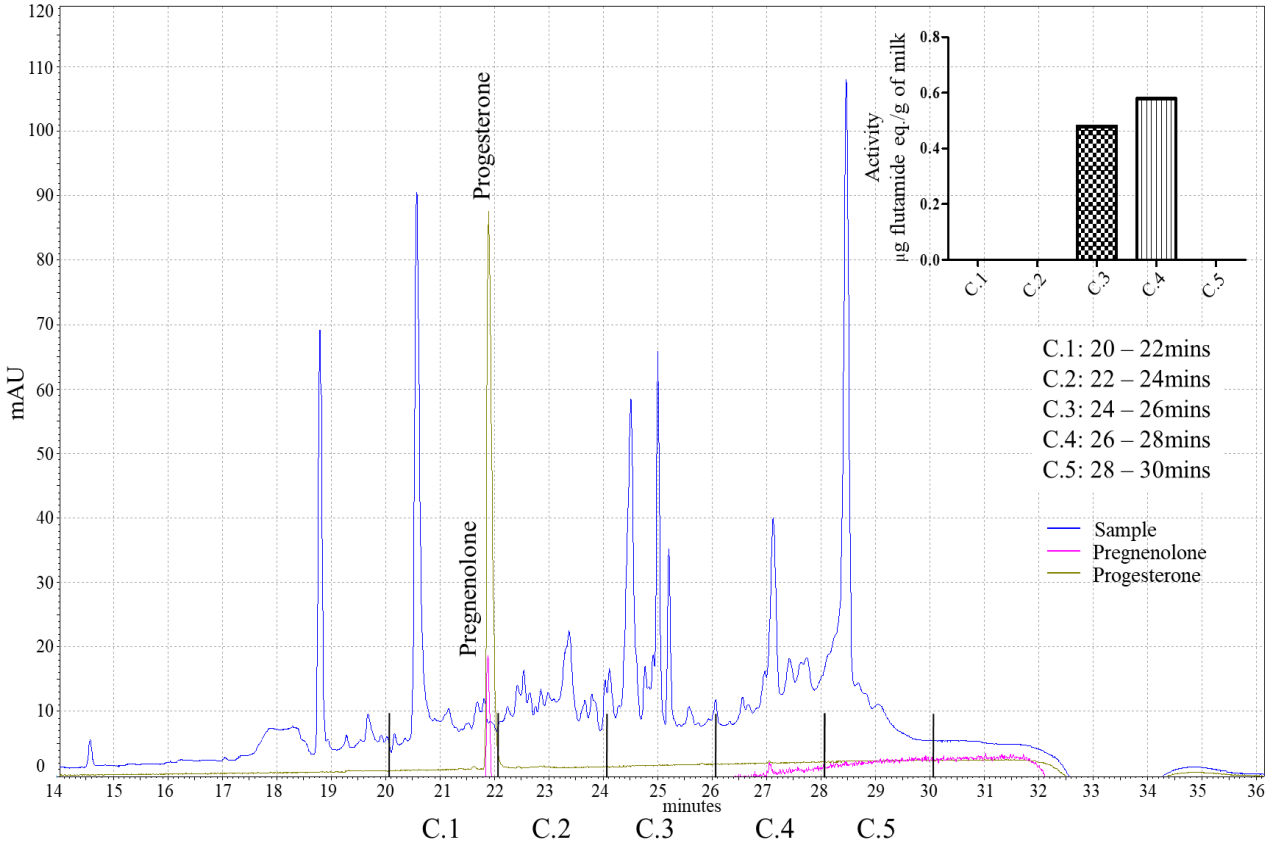

Figure 4. Further refined sub-fractionation of the anti-AR activity containing B.3 fraction on the Phenomenex Kinetex Fluor 5 column, with elution pattern overlay of pregnenolone and progesterone.

Subsequently, a suspect list of 248 human metabolites was generated based on the seven endogenous hormones, set as parent compounds, using a combination of the BioTransformer website and the software R (Appendix A.2). This list was used for the identification of metabolites in C.3 and C.4 fractions after UHPLC-Q-TOF-MS/MS-based non-targeted analysis. After comparison between the predicted masses and the analyzed unique features, none of the metabolites were detected in the fractions of interest showing that neither these compounds nor their parent hormones (androstenedione, DHEA, E1, E2, pregnenolone, progesterone, testosterone) can explain the anti-androgenic activity found in breast milk samples.

Discussion

In this manuscript, we identified anti-androgenic activity in human milk samples derived from the Norwegian HUMIS cohort. Through a chemico-biological analysis, we could exclude that androstenedione, DHEA, E1, E2, pregnenolone, progesterone, testosterone and their metabolites were responsible for the observed anti-androgenic activity measured using the AR CALUX bioassay. While it is not excluded that endogenous hormones might contribute to the anti-androgenic activity observed by the as yet unidentified EDCs, the findings suggest that breastfeeding might constitute a potential route of early human exposure to mixtures of anthropogenic contaminants with anti-androgenic properties.

Excellent correlations were observed between the *in vivo* Hershberger classifications and the AR CALUX results for the sixteen reference compounds selected. Similar to the highly selective ERα CALUX bioassay, the (anti-)AR CALUX reporter gene bioassay may provide a reliable alternative test method to the traditional *in vivo* methods in rodents (Sonneveld et al. 2006). Indeed, the six chemicals, reported as non-active in the Hershberger database established by Browne et al. (2018), scored negative on both AR-based CALUX assays. Testosterone and 17α-methyl testosterone, two natural androgens, shown strong agonistic properties in the AR CALUX bioassay, matching our expectations. These results are consistent with Sonneveld et al. (2006) demonstrating the abilities of the AR CALUX in detecting agonistic activities using a wide array of mainly steroidal compounds, also showing a parallel between *in vitro* and *in vivo* predictions. Moreover, previous studies showed that the assay also responds to all bioactive androgens present on the prohibited list of the World Anti Doping Authority (Houtman et al. 2009). The AR CALUX in its antagonistic mode was, however, never compared to *in vivo* studies. Consequently, a larger set of antagonists was selected. Predictivity of the anti-AR CALUX bioassays was very satisfying and in line with earlier work by Van der Burg et al. (2010). However, one compound, DEHP, scored differently than reported in the Hershberger database. This phthalate was inactive in the *in vitro* anti-AR CALUX assay, which is in line with the results from the ToxCast AR model described by Kleinstreuer et al. (2018) also scoring DEHP negative. In the ToxCast study the highest concentration tested was equal to 100 μM, similar to the concentration used in the anti-AR CALUX. By extrapolating the estimated administered dose, Kleinstreuer et al. showed that this concentration was similar to the lowest observed effect level (LOEL) values derived from the Hershberger method, ranging from 100-200 mg/kg body weight per day, suggesting that the discrepancy in classification between the methods could not be explained by a difference in concentration (Kleinstreuer et al. 2018). The mismatch is very likely caused by the fact that DEHP antagonizes steroid production, which will be measured *in vivo* as an antagonistic

effect, while this is not picked up *in vitro* in a receptor-based assay, but rather in a steroidogenesis assay as present in an EATS panel of assays (Borch et al. 2006). Both ERα- and AR- *in vitro* bioassays showed a good performance and applicability in detecting developmental and reproductive toxicity of chemicals and were found to be powerful tools in predicting the effects of EDCs on the endocrine system (Piersma et al. 2013; Van der Burg et al. 2014).

The pilot epidemiological study including ten breast milk samples revealed an activation of the estrogen receptor α in two extracts only. However, an antagonistic activity towards the androgen receptor was measured in most of the assessed samples. The findings regarding ERα activation are crucial to understand the origins of the measured anti-androgenic activity. Indeed, diverse estrogens such as E1 and E2 were earlier reported to be both androgenic and anti-androgenic, albeit at much higher dosages (more than 1000-fold), levels not reached in normal physiology. The absence of estrogenic activity in most of breast milk extracts could be explained by the typical drop in estrogens levels immediately in the postpartum period, programmed to induce and maintain lactation during the first months after delivery (Lu et al. 2017). This hypothesis is in line with the sampling time of the present samples, ranging from two weeks to two months after birth. Conversely, high levels of estrogens can lead to a reduced milk production resulting in difficulties to breastfeed, which is obviously not relevant for our study group. Further investigations on the anti-AR CALUX showed that the antagonistic activity could be fully attributed to the polar fraction with an average of 4.0 µg flutamide eq./g. On the contrary, all measurements of the apolar fraction were found to be below the limit of quantification. No indication of synergy between the two fractions was noticed. However, it appears that the presence of the apolar fraction, although having a limited response, induces the repression of the activity of the polar extracts when assessed as a mixture (0.58 µg flutamide eq./g on average). Interestingly, this response contrasts with the additive or synergistic effects usually expected from EDCs mixing cocktails (Bergman et al. 2012b; Kortenkamp 2014). While the presence of polar anti-androgenic EDCs could be linked to the activity measured on the anti-AR CALUX bioassay, we cannot exclude the fact that a mixture effect could lead to an enhanced anti-AR activity in the samples. Indeed, studies showed that chemicals with similar anti-androgenic properties may induce strong adverse outcome in male rats, in comparison, only showing small effects were noticed during individual chemical analysis (Hass et al. 2007). Therefore, it is unlikely that the activity measured in breast milk samples could be solely attributed to the presence of certain highly anti-androgenic EDCs whereas, a more complex combination of different type of chemicals is more plausible. Total activity measurements such as the CALUX method gives an overview of

the activity considering all chemicals present in the sample however, further analysis is required to identify a specific chemical to be avoided.

In our samples, we expected natural estrogens and androgens to be extracted along with polar chemicals, thus their potential presence and activity had to be assessed. Using a pooled human milk sample as a basis, we investigated the possible contribution of seven relevant steroids to the measured anti-androgenic activity (Barreiro et al. 2015; Burger 2002). E1, E2, androstenedione, DHEA and testosterone were eluted together as may be expected due to their structural similarities and comparable physico-chemical properties. Elimination of the natural AR agonists, androstenedione, DHEA and testosterone from the extract might explain the observed increase in antagonistic activity, from 0.88 to 1.7 μg flutamide eq./g (Houtman et al. 2009). On the contrary, pregnenolone and progesterone's removal coincided with a drop of about 30% of the activity. Overall an antagonistic activity of 1.1 μg flutamide eq./g was still noticeable after exclusion of the seven endogenous steroid hormones. The remaining anti-AR activity in human milk samples after removal of the sex hormones indicates that other compounds than these natural sex hormones are causative for the observed anti-androgenic activity. It is important to note that, steroid metabolites might also exert an influence on the anti-AR activity, although this seems unlikely since steroidal metabolites do hardly show any remaining receptor-based bioactivities (Houtman et al. 2009). In addition, UHPLC-Q-TOF-MS/MS-based screening of a pooled breast milk sample indicated the absence of 248 potential metabolites derived from our initial set of hormones, which further excluded the hypothesis of involvement of natural hormones or their metabolites in the observed anti-androgenic activity in human milk. Thus, it is more likely that the observed anti-AR activity in human milk samples is caused by yet unidentified anthropogenic, exogenous chemicals which may be present as contaminants. Over the past decades, many studies reported breast milk contamination by various EDC classes. In 1998, Sonawane (1995) established an extensive list of these groups of contaminants describing pesticides (DDT, DDE, dieldrin, aldrin and endrin), polychlorinated biphenyls (PCBs) and dioxins as the most preeminent compounds. The physico-chemical properties of aldrin, dieldrin and endrin imply that these particular pesticides might be present in the polar fraction of breast milk. For *o,p'*-DDT and *p,p'*-DDE which turned out to be potent anti-androgens in the anti-AR CALUX, it is unlikely that they may have contributed to the anti-AR activity in human milk, due to their apolar nature, and therefore they would not likely elute in the polar fraction of our samples where the anti-AR activity was observed (Van der Burg et al. 2010). The same argument goes for dioxins, dioxin-like PCBs and PCBs which can be expected in the apolar extract rather than the polar fractions. While these pollutants are not in the fraction of current interest, their role in the activity measured in the human milk samples should not be excluded. As demonstrated in the previous

section, the apolar fraction, while remaining quite low in anti-AR activity, might still play a role in diminishing the overall activity when measured as a reconstituted sample. Over the years, studies focusing on human milk have identified brominated flame retardants (polybrominated diphenyl ethers (PBDEs) and hexabrominated cyclododecane (HBCD)), dibenzo-p-dioxins (PCDDs), phthalates esters along with other organochlorine pesticides (aldrin, chlordane, α-endosulfan, metoxychlor, etcetera), constituting an non-exhaustive list of potential actors in the measured anti-AR activity (Damgaard et al. 2006; Main et al. 2006, 2007; Norén and Meironyté 2000).

Conclusion

The present manuscript describes a first attempt in understanding the observed anti-androgenic activity in breast milk samples. We found that the anti-AR activity could be distinguished from the major natural steroid hormones, and their metabolites, present in human milk and therefore suggests that the observed anti-AR activity might be associated with yet unidentified contaminants of anthropogenic origin. In future studies, further targeted and untargeted analysis are required to precisely identify their chemical nature. In addition, the possible role of this anti-AR activity in the observed increase in adverse health outcome e.g., frequency of cryptorchidism in offspring from the Norwegian HUMIS cohort will be studied in more detail as well.

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Anti-Androgenic Compounds in Breast Milk and Possible Association with Cryptorchidism Among Norwegian Boys in the HUMIS Birth Cohort

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Abstract

Background The prevalence of cryptorchidism has increased over the past decades, yet its origins remain poorly understood. Testis descent is dependent on androgens and likely affected by endocrine disrupting compounds (EDCs), targeting the androgen receptor (AR).

Objective We investigated the association between anti-androgenic activity, not derived from natural hormones, in maternal breast milk and impaired testis descent among boys.

Methods We performed a case-control study based on 199 breast milk samples from 94 mothers of cryptorchid boys and 105 random non-cryptorchid boys participating in the Norwegian HUMIS (Human Milk Study) cohort. For each participant, apolar, and polar fractions were extracted, and combined to reconstitute a mixture. Anti-androgenic activity was measured in all three fractions using the human cell-based in vitro anti-AR CALUX® assay and expressed in μg of flutamide equivalent, a well-known anti-androgen. Results from fraction analyses were compared among boys with cryptorchidism and controls using multiple logistic regression, controlling for appropriate confounders identified using a directed acyclic graph.

Results Children's daily exposure to anti-androgenic EDCs through breastfeeding was estimated to 78 μg flutamide eq./kg of body weigh/day. The activity was higher in the polar fraction (1.48±1.37 μg flutamide eq./g of milk) representing non-persistent chemicals, in contrast to other fractions. However, the activity in the polar extracts was decreased when in mixtures with the apolar fraction, indicating synergistic interaction. No significant difference in the activity was observed according to cryptorchid status, but we observed a non-significant positive association between anti-androgenic activity in the apolar fraction and cryptorchidism (Odd Ratio = 2.2, 95% CI (0.4, 13.3)).

Discussion The study showed anti-androgenic activity in nearly all human milk samples, and at levels higher than the advisory threshold. A non-significant association between the apolar fraction which represents non-persistent chemicals and cryptorchidism was observed warranting further studies.

Introduction

During the last few decades, an alarming increase in the prevalence of undescended testis in male newborns has been reported (Boisen et al. 2004; Toppari et al. 1996). Cryptorchidism is a risk factor for developing testicular germ cell cancer and impaired fertility, but its etiology is to this date still not fully understood (Mieusset et al. 1995). Over the years, it has been hypothesized that exposure to environmental factors such as endocrine disrupting chemicals (EDCs) could potentially add up to cause uncomplete masculinization in boys exposed during pregnancy (Sharpe and Skakkebaek 1993, 2003; Toppari et al. 1996).

Many consumer products contain EDCs with either weak estrogenic (bisphenol A) or anti-androgenic (phthalate) properties even in relatively low dosages (Sohoni and Sumpter 1998; Bergman et al. 2012). Exposure to EDCs has been associated with various adverse health effects including disruption of the proper masculinization of the gonads (Bergman et al. 2012; Toppari et al. 1996). In experimental animals, impact of EDCs is dependent on the level and period of exposure, identifying fetal and early life as a particularly vulnerable window (Bergman et al. 2012). In humans this window of androgen dependence of testicular decent occurs between 8 to 10 gestational weeks and much later around 25 to 35 gestational weeks (Hutson et al. 2013; Welsh et al. 2008). Some studies have reported associations between prenatal exposure to EDCs e.g. xenoestrogens, polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DTT), and cryptorchidism at birth (Brucker-Davis et al. 2008; Fernandez et al. 2007). However, subsequent studies on cryptorchidism reported that PCBs and DDT placenta levels, as well as perfluorinated compounds cord blood concentrations were not associated with cryptorchidism (Jensen et al. 2014; Virtanen et al. 2012).

To date, most case-control studies associating exposure to EDCs and cryptorchidism are based on analysis of a limited number of chemicals. This is an important shortcoming since it has been shown that the effects caused by EDCs can add up in mixtures (Kortenkamp 2014; Rajapakse et al. 2002). Because most EDCs at realistic exposure levels only show weak binding to nuclear hormone receptors, a more comprehensive analysis of their combined effects is needed to elucidate their possible role in the increased incidence of cryptorchidism. Since the main nuclear receptor involved in male sexual development is the androgen receptor (AR), it can be used as a model to identify EDCs with the potential to be involved mechanistically in cryptorchidism. Breast milk is a suitable bio-fluid for assessing maternal body burden of persistent chemicals accumulated over life, and thus a good indicator of fetal exposure to persistent chemicals during the critical androgen-driven phase of testicular descent (Skaare et al. 1988).

In our previous pilot study, we developed a method to distinguish endogenous hormones from EDCs in human milk samples (Collet et al. 2020). Furthermore, we found anti-androgenic activity in most samples and demonstrated that this activity could not be explained by endogenous hormones present in the milk. The present study therefore investigates the possible association between the disruption of androgen action, through mixtures of chemicals antagonizing normal AR functioning, and cryptorchidism. We extend the previous analyses using the anti-AR CALUX® (Chemically Activated LUciferase gene eXpression) bioassay to evaluate the effects of mixtures of EDCs on AR activity (Sonneveld et al. 2006).

Material and methods

Study population

The study population is a subset of participants from the multi-center birth cohort Norwegian Human Milk Study (HUMIS) previously described in detail (Eggesbø et al. 2009, 2011) (Supplementary Table 1). In brief, HUMIS is a prospective mother-infant pair study performed at the Norwegian Institute of Public Health. Between 2002 and 2009, 2606 mothers were recruited by public health nurses in seven counties across Norway, except for Østfold county where they were recruited by a pediatrician in Østfold hospital. In the present study, 199 mother-child pairs were selected based on the following criteria: singleton and live born. The cases and controls were selected based on the availability of adequate volume of banked breast milk samples for extraction and analysis. For each case, about one potential control was selected by restricting the sex to boys-mother pairs with adequate milk sample for extraction and analysis. Details of the selection procedure are shown in Figure 1.

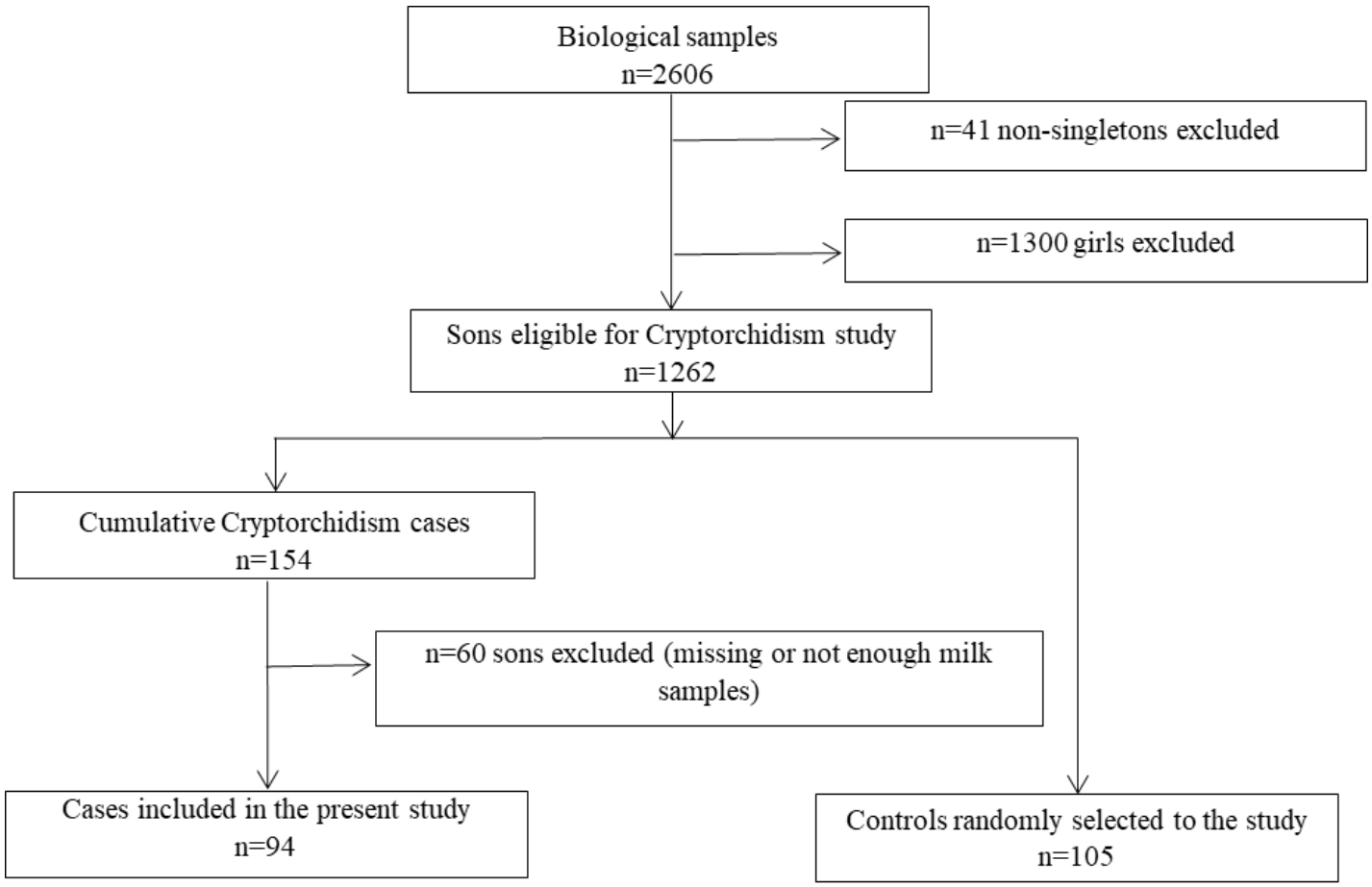

Figure 1. Flow chart of the study population selection for the case-control study.

Cryptorchidism: definition and mapping

Mothers were asked to fill in questionnaires at 1, 6, 12 and 24 months after delivery. The reported number of cryptorchidism cases were 55 at 1 month (n = 55/907), 24 at 6 months (n = 24/986), 24 at 12 months (n = 24/1036), and 26 at 24 months (n = 26/1043). The cumulative reported number of cases at any time point (1, 6, 12, 24 months) was 154 (n = 154/1262). The unique inclusion criteria of the case-study for a mother/child pair was to have a boy with reported cryptorchidism at any time point (1, 6, 12 or 24 months). A total of 94 out of 154 eligible participants fulfilled the inclusion criteria and were selected as cases having enough milk samples for analysis. 105 controls were selected randomly from the eligible dataset (n = 1262) following the same inclusion criteria.

Mother milk collection

Women were asked to collect 25 mL of milk every morning for eight consecutive days between the first two weeks and two months after giving birth. Mothers were encouraged to avoid electrical pumping equipment and were asked to register details on how and when the milk was sampled (Eggesbø et al. 2011). Milk samples were collected in 250 mL natural HDPE Packaging Bottles (Cat. No.: 967-21244, Thermo Scientific Nalgene®) made from food-grade high-purity resins, previously tested for potential migration of antiandrogenic chemicals (Collet et al. 2020). The milk bottles were posted by the mothers and stored at -20 °C in a Biobank of the Norwegian Institute of Public Health upon arrival. In this case-control study we selected milk samples from a subset of mothers to 94 boys with cryptorchidism (unilateral or bilateral) and to 105 boys with no cryptorchidism.

Ethical approval

The study was approved by the Norwegian Data Inspectorate (ref. 2002/1398) and Regional Ethics Committee for Medical Research (ref. S-02122). Mothers were included after oral and written informed consent had been obtained.

Covariates

Questionnaires filled in by the mothers were used to obtain information on potential confounders. Information on the child's sex, birth weight, preterm, gestational age and maternal smoking during pregnancy was obtained by linkage to the Medical Birth Registry of Norway (Skjærven et al. 2000). Maternal age (< 25, 25-35, > 35 years old), pre-pregnancy body mass index (BMI) (under-weight, normal, overweight, obese) and birth weight (< 2500 g, 2500 to 4000 g, > 4000g) were entered as continuous variables. Gestational diabetes, preeclampsia,

parity, preterm (child born before 259 days or 37 completed weeks), small for gestational age (< 10th percentile), smoking habits (no smoking, daily smoker < 10 cigarettes, daily smoker > 10 cigarettes), as well as maternal education (low, medium, high) were set as categorical variables.

Sample preparation

Analytes derived from each breast milk sample were extracted following a two-step extraction method previously described in (Collet et al. 2020). To extract apolar compounds, 5 mL of homogenized breast milk per sample was transferred to a clean 60 ml glass tube with 5 mL of 2-propanol (CAS. no.: 67-63-0, BioSolve) and shaken for 10 min on a shaker at 200±20 strokes per minute. 14 mL of n-hexane (CAS no.: 110-54-3, BioSolve) was added and the tubes were shaken for an extra hour at 200±20 strokes per minute. The upper layer was collected into a clean collecting tube and the procedure was repeated twice with a shorter shaking time (30 min). Collected fractions were evaporated to dryness and reconstituted in 1 mL of n-hexane. Glass columns were prepared with 5 g of 2% deactivated silica and conditioned with 12 mL of n-hexane. Samples were loaded and eluted with 30 mL of a combination of n-hexane and dichloromethane (CAS no.: 75-09-2, BioSolve) to a ratio of 3:1. The final extract was evaporated until dryness, reconstituted in 30 µL of DMSO and stored at -20 °C.

One control consisting of 5 mL of breast milk primarily spiked with 50 µL of a solution of 13C-labeled internal standard containing PCB153L and PCB180L (200 ng/ml) (MBP-D7, Wellington Laboratories) was added to every ten-sample batch. Controls were processed as described earlier (Collet et al. 2020). In brief, controls were extracted following the same procedure as the samples with the exception that the final fraction was reconstituted in isooctane (CAS no.: 540-84-1, BioSolve). Internal standard recoveries were assessed on a gas chromatograph/tandem mass spectrometer (GC-MS/MS) system using gas chromatograph GC-2010 Plus (Shimadzu) and gas chromatograph mass detector GCMS-TQ8050 (Shimadzu) controlled by the program GCMS Real Time Analysis (Shimadzu) and a CTC CombiPal autosampler controlled by the software Cycle Composer (CTC Analytics AG). Parameters and settings used for the analysis were similar to those described by (Collet et al. 2020). Overall, recovery values were 96±13 and 115±21% for PCB153 and PCB180, respectively.

Polar compounds from breast milk samples were extracted according to the method detailed by (Collet et al. 2020). In brief, we adapted and optimized a QuEChERS (Quick Easy Cheap Effective Rugged and Safe) solid phase extraction and cleaning method for isolating polar EACs in the breast milk samples (Anastassiades et al. 2003). The 199 samples were homogenized prior to the procedure. In a clean 50 mL tube (Grenier Bio-One), 5 mL of sample

was transferred and completed with 15 mL of acetonitrile (ACN) (CAS no.: 75-05-8, BioSolve). After 30 secs of vigorous shaking, one QuEChERS EN 15662 extraction packet (Cat. no.: 5982-5650, Agilent) was added. Tubes were shaken for 15 min using a circular shaker and then centrifuged for 5 min at 4000 rpm at 4 °C. The upper phase was collected in a clean 60 mL glass tube and the solid lower phase mainly constituted of salts was re-dissolved by adding 15 mL of ACN. The complete procedure was repeated once using the same Grenier tube but without adding sample. The upper layers were combined and transferred to a 15 mL QuEChERS d-SPE (Cat. no.: 5982-5158, Agilent) clean-up tube. After 1 min vortex for homogenization, the d-SPE tubes were centrifuged for 5 min at 4000 rpm at 4 °C. Subsequently, the upper layer was collected and evaporated until dryness. All extracts were reconstituted in 30 µL of DMSO and stored at -20 °C until analysis.

Samples were extracted by sets of 10. A procedure control consisting of 5 mL of spiked breast milk was included to each batch to assess the efficiency of the extraction. 100 µL of a mixture of internal standards (100 µg/mL) of bisphenol A (CAS no.: 80-05-7, Sigma-Aldrich), 17β-estradiol (E2) (CAS no.: 50-28-2, Sigma-Aldrich) and testosterone (CAS no.: 58-22-0, Sigma-Aldrich) was used, similar to the pilot study (Collet et al. 2020). Controls were extracted following the same procedure as the samples with the exception that the final extract was reconstituted in pure ACN. Controls were analyzed by liquid chromatography using a Kinetex Biphenyl column (150x4.6mm 2.6µ particle size) (Cat. no.: 00F-4622-E0, Phenomenex). The system setup was the same as detailed earlier (Collet et al. 2020). After analysis, recovery values were calculated by comparing the peak height in the control samples with the initial internal standard spiking solution. BPA, E2 and testosterone were recovered to a rate of 35±13, 50±6.8 and 59±5.9%, respectively. The polar fraction was compensated for loss i.e. measurements were adjusted using a factor of 2. Moreover, for the reconstituted mixture, the combination of polar and apolar (originally 1:1 ratio) was adapted to insure the correctness of the study (2:1).

Anti-AR CALUX bioassay

Apolar, polar and mixed fractions derived from each sample were individually analyzed on the anti-AR CALUX reporter gene assay (Sonneveld et al. 2005). The anti-AR CALUX assay is based on human osteoblastic osteosarcoma U2-OS cell-line (American Type Culture Collection), cultured as described previously (Sonneveld et al. 2005). The AR cell line is stably transfected with a full-length human AR expression vector and a minimal promoter element coupled to a luciferase reporter construct containing three androgen responsive elements (Sonneveld et al. 2005).

Blinded analysis was chosen to reduce bias. Anti-AR CALUX analysis was performed using a Hamilton Starlet liquid handling robot coupled to a Cytomat incubator. The procedure was adapted from the automated version of the CALUX bioassay described earlier in Van der Burg et al. (2014). For this, cultured AR CALUX cells were re-suspended in assay medium ($1x10^5$ cells/mL) consisting of DMEM/F12 medium without phenol red indicator (Cat no.: VX1041025, Fisher) supplemented with 10 U/mL penicillin and 10 µg/mL streptomycin (P/S), non-essential amino acids (NEAA) (Cat no.: 11140-03, Gibco) and 5% charcoal-stripped fetal calf serum (DCC). 100 µL were transferred in 96 well plates and incubated for 20±4 hours at 37 °C and 5% CO_2. After incubation, samples were placed in the handling robot along with a nine-point calibration line of the reference compound flutamide ranging from 0M, consisting of pure DMSO, to $1.0E^{-05}$ M (stock solution). The robot was programmed to dilute each extract (dilution series 1-3-10-30-100x) using assay medium supplemented with $3.0E^{-10}$M of 5α-dihydrotestosterone i.e. DHT (CAS: 521-18-6, Sigma Aldrich). The final DMSO concentration was set to 0.2%. The robot was programmed to fill the 96-plate with 100 µL of exposure medium. All dilution points including the calibration line were tested in triplicate on the same 96-well plate. A solvent control (DMSO) was added to each plate to evaluate background activity. After 22±2 hours of incubation the medium was discarded and replaced by 30 µL of a Triton-lysis buffer. Plates were shaken for 10 minutes and the luciferase signal in cellular lysates was measured using a Tristar luminometer (Berthold).

Controls

All anti-androgenic measurements were performed along with the Cytotox CALUX® assay. Used as a control, the assay identifies non-specific luciferase activity repression e.g. caused by cellular death. The Cytotox CALUX bioassay consists of U2-OS cells constitutively expressing the luciferase gene (Van der Linden et al. 2014) and was essentially performed as described above with the exception that the analysis was done by hand with undiluted samples. A full dose response curve of the reference compound tributyltin acetate (CAS: 56-36-0, Merck Chemicals B.V.) was added to each Cytotox CALUX plate. Samples inducing a decrease in luminescence of more than 20% (i.e. cytotoxicity ≥ 20%) were further diluted and reanalysed. For further evaluation of non-specific signals, ten breast milk samples randomly selected from the sample set were incubated with an excess amount of DHT (i.e. 1000-times the EC50 concentration). DHT saturating concentration is set to maintain receptor activation through competing with anti-androgenic compounds. In this way, a remaining decrease in luminescence demonstrates a non-specific repression, independent from AR inhibition. As none of the tested samples showed such repression of the signal, further measurements were considered as true antagonistic activity.

Data handling

For each plate, the EC50 DHT agonistic signal was set to 100% and the maximum signal response induced by the antagonist flutamide was defined as 0% (Figure 2). Relative Light Units (RLUs) measurements were corrected for background using DMSO. The average of each triplicate was plotted to the reference dose-response curve. Subsequently, all results were transformed using the statistical software package GraphPad Prism 5.0 (non-linear regression, variable slope, 4 parameters, robust fit), and expressed as a percentage of the maximum signal (relative induction). The relative induction was used to quantify the anti-androgenic activity in each sample expressed as µg equivalent of flutamide per gram of milk (µg flutamide eq./g of milk).

A sample is considered active if its activity surpasses the limit of quantification (LOQ) of the anti-AR CALUX bioassay, set to ≥ 0.40 µg flutamide eq./g of milk. A non-active sample i.e. activity ≤ LOQ, was replaced by an estimation of the activity equal to half the LOQ value. CALUX measurements were evaluated according to the following criteria: EC50 of the reference compound between assay-specific predetermined limit values; R2 of standard curve > 0.98; z-factor of standard curve > 0.6.

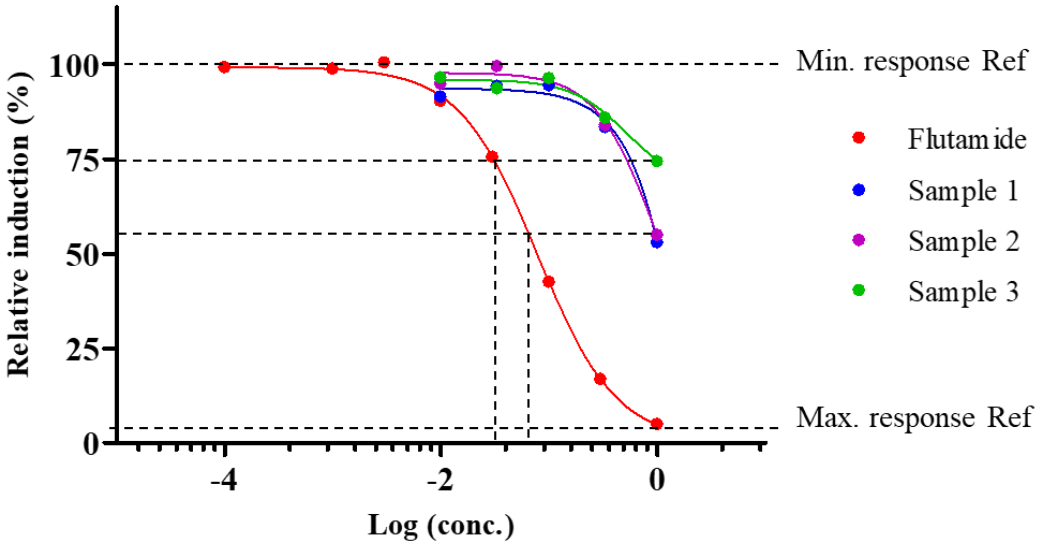

Figure 2. Example of the antagonistic evaluation method for three breast milk samples using flutamide as a reference compound.

Statistical analysis

Differences in maternal and child characteristics between cryptorchidism cases and controls in our study were tested in a univariate analysis using Pearson's chi-square test for

binary or categorical variables, and the Wilcoxon rank-sum test for the continuous variable (gestational age).

Adjustment models. To estimate the association between anti-AR activity in human milk samples and the risk of cryptorchidism, appropriate confounders were first identified using a Direct Acyclic Graph (DAG) (DAGitty v3.0) (Figure 3). Multiple logistic regression models were used to estimate adjusted Odds Ratio (OR) and 95% Confidence Interval (95% CI). Two sets of potential confounders were identified. Set 1 included socioeconomic status (SES), maternal age, pre-pregnancy BMI, preterm, gestational age and parity, in which the overall percentage of missing values was 7%. Set 2 included small for gestational age, gestational diabetes, preeclampsia, smoking during pregnancy, alcohol consumption during pregnancy, antibiotic use, fat content of the milk, and a selected maternal toxicant. The overall missing when adjusting for both set 1 and 2 was 83% (Supplementary Table 2). The high percentage of missing was due to the fact that information on some covariates were obtained through linkage with the Norwegian MoBa study, for the small set of participants who also participated in the MoBa study. However, the high percentage of missing values in the fully adjusted model was problematic, so we decided to use multiple imputation by predictive mean matching in Stata as a sensitivity analysis. As a result, the other set of potential confounders (alcohol use, smoking, antibiotics use, maternal toxicant and fat content) were dropped since they had high missing values, and sensitivity analysis after multiple imputation by predictive mean did not change the result (Supp. Fig 1-5). The final model was therefore a complete case analysis of the association between anti-AR activity measured in breast milk fractions (polar, apolar, mixture) and the risk of cryptorchidism adjusted for variables in set 1.

Software. Stata (version 16.1; Stata Corp LP, College Station, Texas, USA) was used for all statistical analyses. Statistical significance was set up at $p < 0.05$.

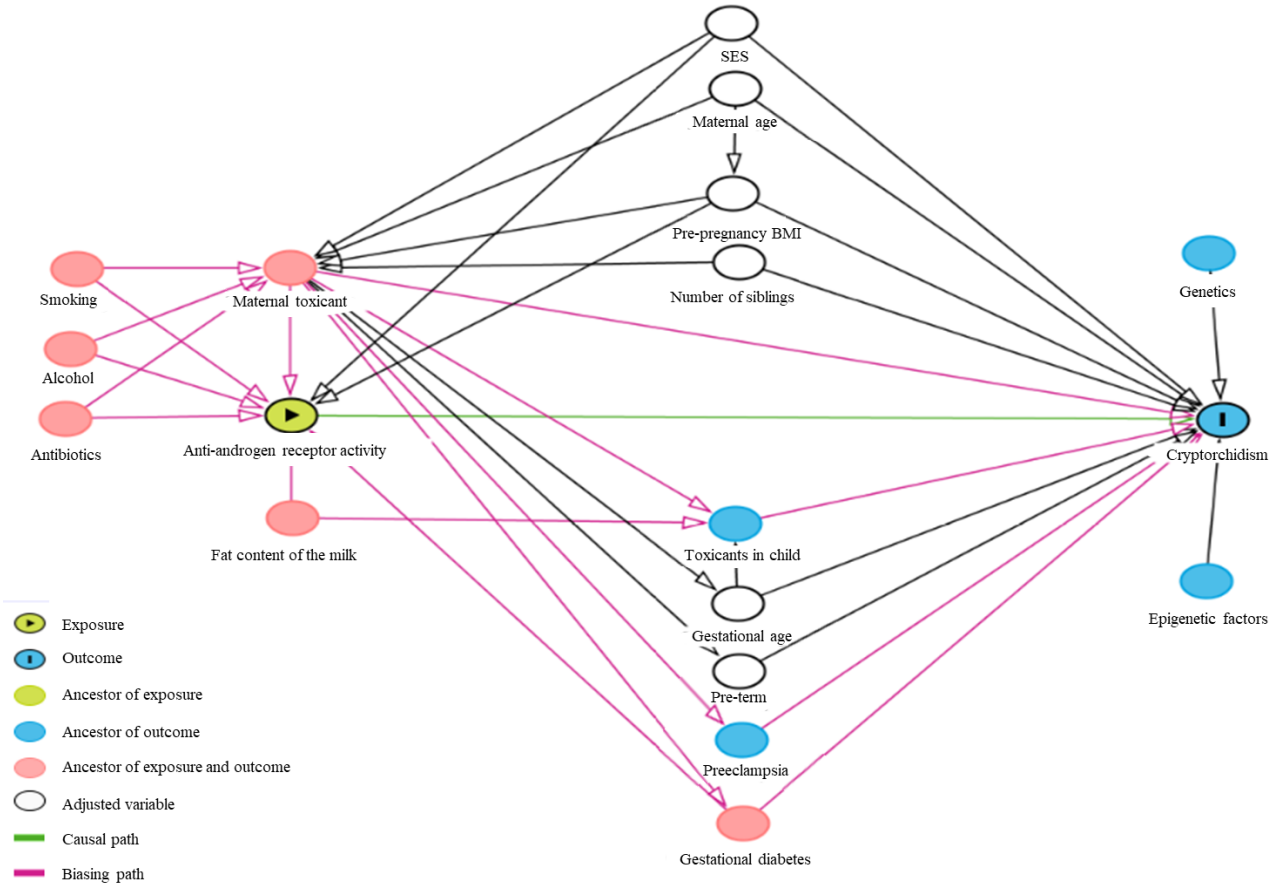

Figure 3. Directed acyclic graph (DAG) based on literature for the associations between anti-AR activity measured in breast milk (exposures), covariates, and cryptorchidism among Norwegian sons in the HUMIS study.

Note: The first modelling set (S1) included relationship with maternal factor marked as adjusted on the DAG (White circle). The second modelling set (S2) included more potential confounders and were shown as unadjusted on the DAG (red circle). The DAG was created using DAGitty. http://www.dagitty.net/dags.html

Results

The characteristics of the study population in which anti-androgenic activity was assessed are presented in Table 1. Of 199 mothers, 148 (74.4%) were between 25-35 years of age at delivery, comparable between cases and controls. Most of the participants were highly educated (75.4%). The study population shows a minority of overweight or obese women (31%) and more than 80% reported no smoking history. In both cases and controls, most boys weighted within 2500-4000 grams at birth. A non-significant tendency of lower education among mothers of cases was observed. Overall, no major difference was found between the mothers giving birth to a cryptorchid boy versus a healthy child. Also, the selection of the case-control study participants is representative of the study population enrolled in the HUMIS cohort with respect to maternal age, maternal education, and small birthweight for gestational age (9.5% vs 10%). Preterm was lower in the present study compared to the study population (6.5% vs 9%). Detail of the profile is presented in the supplementary material (Table S1).

Table 1. Univariate analysis of the association between maternal and child characteristics of the study participants (n (%) or median (IQR)) and the occurrence of cryptorchidism in a case-control study among 199 mother-child pairs, Norway.

Characteristic	Total n=199	Controls n=105	Cases (cryptorchidism) n=94	*p-value*
Maternal age (years)				
<25	30 (15.1%)	15 (14.3%)	15 (16.0%)	*0.94*
25-35	148 (74.4%)	79 (75.2%)	69 (73.4%)	
>35	21 (10.6%)	11 (10.5%)	10 (10.6%)	
SES				
Low	19 (9.5%)	7 (6.7%)	12 (12.8%)	*0.16*
Medium	26 (13.1%)	11 (10.5%)	15 (16.0%)	
High	150 (75.4%)	84 (80.0%)	66 (70.2%)	
Missing	4 (2.0%)	3 (2.9%)	1 (1.1%)	
Birth weight (grams)				
<2500	5 (2.5%)	3 (2.9%)	2 (2.1%)	*0.62*
2500-4000	146 (73.4%)	74 (70.5%)	72 (76.6%)	
> 4000	48 (24.1%)	28 (26.7%)	20 (21.3%)	
Parity	78 (40.0%)	43 (41.7%)	35 (38.0%)	*0.60*
Small for gestational age	19 (9.5%)	9 (8.6%)	10 (10.6%)	*0.62*
Gestational age (days)	281.5 (273-288)	284 (272-289)	280 (273-287)	*0.18*
Preterm (before 37 week/259 d)	13 (6.5%)	8 (7.6%)	5 (5.3%)	*0.51*
Pre Pregnancy BMI				
Under weight (< 18.5)	4 (2.0%)	4 (3.8%)	0 (0.0%)	*0.27*
Normal (18.5–24.9)	125 (62.8%)	65 (61.9%)	60 (63.8%)	
Overweight (25–29.9)	39 (19.6%)	19 (18.1%)	20 (21.3%)	
Obese (≥30)	22 (11.1%)	12 (11.4%)	10 (10.6%)	
Missing	9 (4.5%)	5 (4.8%)	4 (4.3%)	
Smoking at the end of pregnancy				
No smoking	160 (80.4%)	83 (79.0%)	77 (81.9%)	*0.55*
Daily smoker < 10	5 (2.5%)	2 (1.9%)	3 (3.2%)	
Daily smoker >10	1 (0.5%)	1 (1.0%)	0 (0.0%)	
Missing	33 (16.6%)	19 (18.1%)	14 (14.9%)	
Preeclampsia	7 (3.5%)	2 (1.9%)	5 (5.3%)	*0.19*
Gestational diabetes	1 (0.5%)	1 (1.0%)	0 (0.0%)	*0.34*

Note: BMI: body mass index; SES: socio-economic status; IQR: interquartile range.

Anti-androgenic activity was evaluated in 199 breast milk samples and expressed as µg equivalent of the reference compound flutamide, per gram of milk. For each participant, the apolar and polar fractions including the endogenous hormones, and the combination of both have been analyzed separately using the anti-AR CALUX bioassay (Figure 4, Table 2.).

Figure 4 and Table 2 present the distribution of anti-androgenic activity in the apolar and polar fractions along with the mixtures of both. The mixtures showed antagonistic properties ranging from 0.40 to 2.82 µg flutamide eq./g of milk. In nine milk samples, the mixtures were found to be ≥ 1.0 µg flutamide eq./g. of milk while in 70 samples, the mixtures were below LOQ (data not shown). Polar analyte-based measurements ranged from 0.41 to 9.25 µg flutamide eq./g of milk, and most samples (124) were above ≥ 1.0 µg flutamide eq./g of milk. In contrast, apolar fractions showed a smaller range of values (0.40 to 1.16 µg flutamide eq./g of milk) with 23 milk samples eliciting activity above the LOQ cut-off value, and only one sample scoring above 1.0 µg flutamide eq./g of milk. Yet, considering that more than half of breast milk samples showed anti-androgenic activity when the mixture was assessed, we estimated the potential daily exposure to anti-androgenic EDCs of a breastfed child during the first twelve months of life.

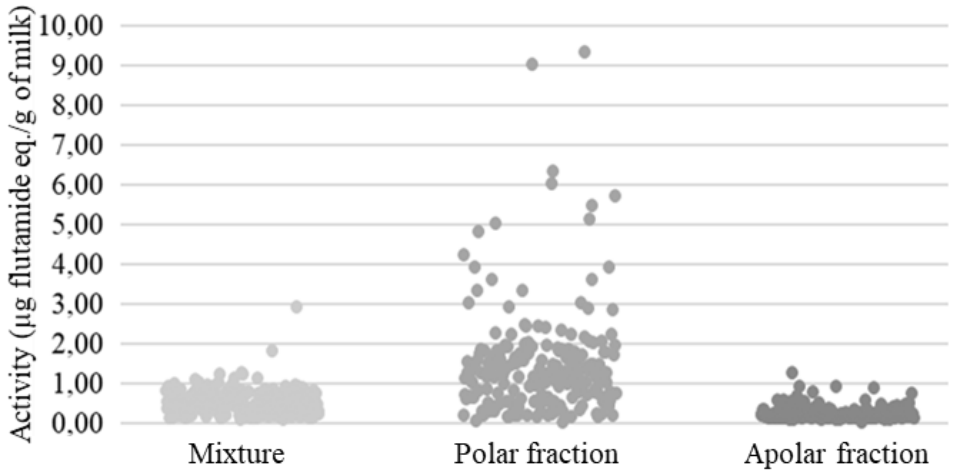

Figure 4. Distribution of anti-androgenic activity in reconstituted breast milk samples (mixture), as well as polar and apolar fractions, measured via the anti-AR CALUX bioassay.

Table 2. Distribution of anti-AR activity in polar, apolar, and combined fractions in breast milk from mothers of cryptorchidism 94 cases and 105 controls enrolled in the Norwegian HUMIS study.

	N	Mean	SD	p5	p25	p50	p75	p95	Max	Range	IQR
Mixtures											
All	199	0.52	0.32	0.13	0.27	0.50	0.70	0.98	2.90	2.82	0.43
Cryptorchidism	94	0.51	0.28	0.14	0.26	0.51	0.68	0.98	1.8	1.68	0.42
Controls	105	0.53	0.36	0.13	0.27	0.48	0.71	0.97	2.90	2.82	0.44
Polar fraction											
All	199	1.48	1.37	0.18	0.64	1.24	1.80	4.20	9.30	9.25	1.16
Cryptorchidism	94	1.49	1.42	0.19	0.64	1.24	1.78	5.1	9.3	9.16	1.14
Controls	105	1.47	1.33	0.17	0.64	1.14	1.80	3.90	9.00	8.95	1.16
Apolar fraction											
All	199	0.22	0.16	0.10	0.14	0.17	0.23	0.55	1.23	1.16	0.09
Cryptorchidism	94	0.23	0.18	0.1	0.14	0.17	0.23	0.57	1.23	1.16	0.1
Controls	105	0.22	0.14	0.10	0.14	0.17	0.23	0.50	0.90	0.82	0.09

Note: Concentration are expressed in µg flutamide eq./g of milk. N: number of samples per category; Mean: average activities; SD: standard deviation; p5-95: 5th to the 95th percentile; Max: maximum activity measured for the category; Range: difference between minimum and maximum activity measured for the category; IQR: interquartile range, difference between p75 and p25.

According to WHO guidelines on breastfeeding (WHO 2011), a child should be exclusively breastfed for the first six months to one year of life. During this period, an infant ingests about 150 mL of milk per kg of body weight (bw) per day, on average. Our previous results showed that breast milk from Norwegian mothers of the HUMIS cohort could be contaminated with anti-androgenic toxicants, equivalent to 0.52 µg flutamide eq./g of milk, on average. Considering these data, and the volume of daily intake of an infant, we estimated the average exposure to anti-androgenic EDCs during the first year of life, in µg of flutamide/kg bw/day (Table 3).

Table 3. Estimation of a child's daily intake of anti-androgenic EDCs through breastfeeding at one month, six months and twelve months.

Average activity (µg flutamide eq./mL)	Age (months)	Daily intake of milk* (mL)	Estimated intake per day (µg flutamide eq./day)	Body weight** (kg)	Nursing child dose per day (µg flutamide eq./kg bw/day)
0.52	1	525	273	3.5	78
0.52	6	1125	585	7.5	78
0.52	12	1380	718	9.2	78

Note: *Assuming that the child is exclusively breastfed.
** Average body weight at 1, 6 or 12 months.

The distribution of anti-androgenic activity in control and cryptorchid groups is shown in Table 2. The mean of the mixed fractions was 0.53±0.36 and 0.51±0.28 µg flutamide eq./g of milk in controls and cases, respectively. Most apolar fractions fell below the LOQ value of the anti-AR CALUX bioassay regardless of the studied group (p75 = 0.23 µg flutamide eq./g of milk). Finally, the mean activities of the polar fractions were 1.47±1.33 and 1.49±1.42 µg flutamide eq./g of milk. In conclusion, the anti-androgenic activity measured in breast milk samples from controls and cases were similar across all the tested fractions. The association between anti-AR activity measured in breast milk fractions (polar, apolar, mixed) and the occurrence of cryptorchidism was estimated using logistic regression (Figure 5, Supp. Table 2). The minimally adjusted model contained maternal age, maternal education, pre-pregnancy BMI, gestational age, and number of siblings, while the fully adjusted model also contained gestational diabetes and preeclampsia. The other set of variables (alcohol use, smoking, antibiotics use, maternal toxicant and fat content) were dropped since they had high missing, and sensitivity analysis after multiple imputation by predictive mean didn't change the result (Supp. Fig 1-5). No statistically significant association was observed between anti-androgenic activity in the mixture or polar fractions and cryptorchidism in the minimal and fully adjusted models. Yet non-significant positive association between anti-androgenic activity in the apolar fraction and cryptorchidism (Odd Ratio = 2.2, 95%CI (0.4, 13.3)) was found.

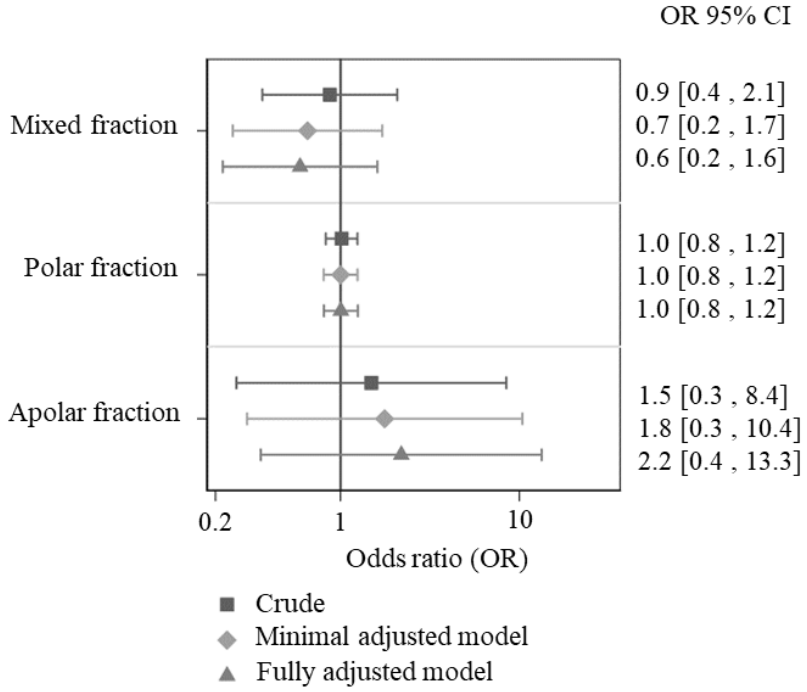

Figure 5. Forest plot showing association between cryptorchidism at one month and anti-AR activity from mixed, polar and apolar fraction of breast milk in HUMIS cohort.

Note: Minimal adjusted model: Maternal age, socio economic status, pre-pregnancy BMI, and parity. Fully adjusted model: gestational diabetes and preeclampsia included in addition.

Discussion

This study shows that nearly all Norwegian babies are exposed to anti-androgenic activity through breast milk and at levels higher than the advisory threshold. Children's daily exposure to anti-androgenic EDCs through breastfeeding was estimated to 78 µg flutamide eq./kg of body weigh/day. A non-significant association between the anti-androgenic activity in the apolar fraction and cryptorchidism was observed in this study, warranting further studies on EDCs and cryptorchidism. No significant association between anti-androgenicity from polar contaminants and cryptorchidism was found, nor from the overall reconstituted samples (mixtures).

Anti-AR CALUX mother milk analysis showed that anti-androgenic activity was associated with the presence of polar compounds in the samples. In contrast, apolar fractions gave a lower anti-AR activity signal. This was unexpected, since the latter fraction is supposed to contain various toxicants such as DDT, DDE, brominated flame retardants, and organochlorines, known to exhibit anti-androgenic activity (Kelce et al. 1995; Lemaire et al. 2004; Van der Burg et al. 2010). Yet, the present results match our previous pilot study demonstrating less activity in apolar extracts in comparison with polar measurements (Collet et al. 2020). Due to their resistance to degradation and extended half-life, persistent toxicants are able to bioaccumulate throughout life in diverse fatty tissues, including breasts. Over the past decades, many studies highlighted EDCs presence in mother milk, demonstrating breastfeeding as an important source of exposure for the infant (Norén and Meironyté 2000; Sonawane 1995; Thomsen et al. 2010). The present study provides insights regarding child's early life exposure showing an almost ubiquitous anti-androgenicity in breast milk. Although generally less potent, apolar fraction analysis provides a better general insight of perinatal exposure in opposition to the polar fraction, which mainly reflects the action of non-persistent compounds i.e. less stable exposure. Yet, exposure may be stable if mothers have a stable consumption/use of products containing non-persistent chemicals. Nevertheless, reconstituted samples analysis highlighted the importance of studying both fractions, alone and in combination. Anti-AR CALUX analysis showed a decline in anti-androgenic activity from polar compounds (p50 = 1.80 µg flutamide eq./g of milk) when assessed along with apolar chemicals as a mixture (p50 = 0.50 µg flutamide eq./g of milk). These results demonstrate that the presence of apolar compounds influences anti-androgenicity by weakening stronger polar chemical effects. While various classes of lipophilic chemicals were proven to cause anti-androgenic activity, there are likely many other chemicals in this fraction, with unknown effect on the AR which could cause unpredicted mixture effects (Bergman et al. 2012; Kortenkamp 2014). Overall, these anti-AR CALUX measurements provide an overview of the AR activity present in human milk. A nursing child's

dose exposure to anti-AR EDCs based on the AR activity levels present in human milk was estimated to 78 μg of flutamide eq./kg bw/day approximately. This level of anti-androgens, expressed as flutamide equivalent, largely exceeds the acceptable daily doses of a flutamide exposure for humans reported in 2017 (no adverse effects level NOAEL of 0.025 mg/kg/day) (Zacharia 2017). Additional studies involving targeted methods and mixture effect analysis are necessary to identify specific compounds and their origins in order to advice regulatory authorities on necessary further chemical restrictions. Nevertheless, the present extraction and analysis of 199 breast milk samples gave a starting point in the path of exploring anti-androgenicity and mixture effect in human milk.

As a first attempt to evaluate the potential risks of an early exposure to anti-androgenic EDCs, we investigated the potential link between anti-androgenic activity in breast milk and cryptorchidism occurrence in the offspring. Anti-androgenic measurements in cryptorchid and control groups were similar regardless of the assessed fraction. Nevertheless, statistical analysis suggests a non-significant tendency for a positive association between apolar anti-androgenic activity and cryptorchidism only. Experimental studies on animals clearly demonstrated that cryptorchidism can be caused by the disruption of hormones involved in regulation of testicular descent (MacLeod et al. 2010; Welsh et al. 2008). The mechanistic studies investigating anti-androgenic activity and cryptorchidism in humans were mainly focused on the relationship between hormone (testosterone/estrogen) levels or non-persistent i.e. polar compounds and cryptorchidism. (Key et al. 1996; Virtanen and Adamsson 2012). Still, there are a few studies investigating on cryptorchidism incidence and apolar chemicals. In 2007, a Danish-Finnish case control study among 280 boys suggested a possible association between flame retardants (polybrominated diphenyl ethers) levels in breast milk and congenital cryptorchidism (Main et al. 2007). Then, Brucker-Davis et al. (2008) showed that cryptorchid boys were more likely to present higher levels of DDE and polychlorinated biphenyls in colostrum in comparison with the control group. While these studies point towards the direction of our results i.e. a potential link between lipophilic compounds and undescended testis, we cannot exclude that the trend we observed in the present analysis could be influenced by the rather low activity derived from apolar anti-AR CALUX measurements, close to the background value of the bioassay. In this way, further investigations combining targeted and untargeted methods, such as CALUX reporter gene bioassays, are necessary to further investigate potential link between chemicals exposure and the increasing cryptorchidism incidence.

This study is the first to investigate anti-androgenic receptor activity in human breast milk fractions in a case-control study selected from prospective cohort. However, some

strengths and limitations need addressing. Firstly, the selection of the study participants in this case-control study compared to the entire cohort is representative with respect to maternal (age, education, parity, pre-pregnancy BMI) and child (gestational age, small for gestational age) characteristics. The HUMIS cohort is a multi-center birth cohort enrolling participants across Norway to represent Norwegian population. The exposure, breast milk extracts were collected after the outcome, however, represents real-life exposure covering both prenatal and postnatal windows especially for lipophilic compounds as it accumulates in lipid-rich tissues and represents mothers body burden. There is also a positive correlation between levels measured in breast milk and levels in the umbilical cord for persistent chemicals making it a suitable proxy for prenatal exposure (Kanja et al. 1992; Verner et al. 2013; Waliszewski et al. 2001). Moreover, using CALUX bioassays and total EDC content, this study takes into account the interactions among "real" chemical mixtures in the breast milk. In addition, we had information on a large set of potential confounders and the outcome, cryptorchidism, was based on repeated questionnaire (maternal reports) at 1, 6, 12, and 24 months which enables to capture recurrent cryptorchidism; normally descended testis at birth that subsequently ascended. While self-report is prone to bias and not as robust as clinically diagnosed cases of cryptorchidism, high reliability has been demonstrated in maternal reports from Norwegian mothers (Skulstad et al. 2017). One of the major limitations was the sample size (199 samples) that might have underpowered the study, preventing the detection of any significant difference. Breast milk was used as a proxy for prenatal chemical exposure which is not suitable for polar chemicals. While it is well-known that apolar compounds bioaccumulate in breast tissues, polar chemicals, i.e. non-persistent compounds, content tends to fluctuate during life. Although polar activity measurements were corrected for loss during the extraction procedure, an underrepresentation of non-persistent EDCs cannot be ruled out. Androgen-dependent phases of testis descent occur during early fetal development (8-10weeks) and much later around 25-35 gestational weeks (Hutson et al. 2013; Welsh et al. 2008). Whereas breast milk appears as a suitable matrix for monitoring late fetal environmental, earlier steps of cryptorchidism determinism might have been overlooked.

In conclusion, in this case-control study we found the ubiquitous anti-androgenicity present in breast milk samples from Norwegian mothers. We highlighted the interactions between chemicals of different polarity and the interest in assessing them alone and in combination. No association between anti-androgenic activity in mother milk and the occurrence of cryptorchidism in the offspring was found, however, a possible association with anti-AR activity from apolar compounds was suggested. Additional studies based on a larger sample size are needed to further explore the relationship between anti-androgenic activity in milk and the risk of cryptorchidism in humans.

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Evaluation of A Panel of *In Vitro* Methods for Assessing Thyroid Receptor B and Transthyretin Transporter Disrupting Activities

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Abstract

We developed a thyroid testing panel to assess endocrine disrupting chemicals (EDCs) capacities to bind either the thyroid receptor β (TRβ) or the thyroid hormones transporter transthyretin (TTR). We first stably transfected a human U2OS cell line with TRβ and a luciferase reporter construct to develop the TRβ CALUX® reporter gene assay to assess chemicals' potential to interact with TRβ. Secondly, we combined a TTR-binding assay with the TRβ CALUX (TTR-TRβ CALUX) and optimized the system to evaluate the competitive properties of EDCs towards T4 for TTR binding. Both systems were evaluated with a range of known thyroid-disrupting compounds. The agonistic/antagonistic TRβ CALUX successfully predicted 9/9 and 9/12 test compounds, respectively. The TTR-TRβ CALUX predicted 9/9 compounds and demonstrated competitive activities when analyzing wastewater samples. We concluded that the proposed test battery is a promising screening method able to efficiently generate data on thyroid hormone interferences by chemicals.

Introduction

Over the course of several decades, some evidence has suggested the sensitivity of fetal development and adult physiology towards endocrine disrupting chemicals (EDCs) (Kabir et al. 2015). It has been reported that a repeated exposure to these EDCs can interfere with endogenous hormones leading to adverse health effects, even at very low concentrations (Kabir et al. 2015). The importance of the endocrine system in major physiological processes and disease etiology emphasizes the growing need of developing reliable high throughput tools to efficiently predict the disrupting activity of chemicals and their mixtures. In the past, we developed CALUX® (Chemically Activated LUciferase eXpression) reporter gene assays able to assess androgen receptor (AR) and estrogen receptor (ER) alterations by environmental chemicals (Sonneveld et al. 2005; Van der Burg et al. 2014). These bioassays cover a variety of endpoints related to EATS (Estrogens, Androgens, Thyroid, and Steroidogenesis) adverse outcomes. In order to further expand our current testing panel, we selected the thyroid system as a next step in developing a complete *in vitro* test battery for assessing developmental and reproductive toxicity (DART) effects.

Thyroid hormones (THs) are important in numerous physiological processes such as regulation of metabolism, bone remodeling, cardiac function and mental status. The two main THs, the prohormone 5′,3′,5,3-tetraiodo-[L]-thyronine (thyroxine, T4) and its bioactive form the 3′,5,3-triiodo-[L]-thyronine (triiodothyronine, T3), play a major role in maintaining body homeostasis (Brent 2012). Thyroid hormonal output is regulated by a finely tuned feedback system acting at both hypothalamic and pituitary levels (Brent 2012; Mullur et al. 2014). First, the thyroid-releasing hormone (TRH) is secreted by the hypothalamus into blood, through the hypophyseal portal system. Once in the anterior pituitary, TRH induces the production of thyroid-stimulating hormone (TSH) which is released in the general circulation towards the thyroid gland. Within the thyroid gland, TSH stimulates the synthesis and secretion of THs. Depending on serum levels, THs can modulate their own production and release by exerting a retro-negative control on both TRH and TSH (Brent 2012; Mullur et al. 2014). Once in the bloodstream, T4, the major thyroid hormone produced, binds to transport proteins so as to be distributed to target cells across the body. In humans there are three major serum carrier proteins, thyroxine-binding globulin (TBG), transthyretin (TTR) and human serum albumin (HSA) (Landers et al. 2013; van der Spek et al. 2017). Although the major transporter is TBG, specific structural properties allow TTR to pass through the blood-brain barrier and the uterine-placental wall making this protein the main carrier of T4 in the cerebrospinal fluid and the developing fetus (Landers et al. 2013). Within the target tissues, T4 is mainly converted into its bioactive form T3 by deiodinase enzymes (Mullur et al. 2014; van der Spek et al. 2017).

Both T4 and T3 are natural ligands for the thyroid receptors α and β (TRα and TRβ) (TRs), with T3 having a 10-fold higher affinity (Kim and Cheng 2013; Ortiga-Carvalho et al. 2014). TRs primary isoforms are differentially expressed in fetal and adult tissues, TRα being predominant in the brain, heart ventricles, intestine and skeletal muscle while TRβ is more widely distributed (brain, retina, inner ear, heart, kidney, liver and lung) (Brent 2012; Ortiga-Carvalho et al. 2014). In 2014, Ortiga-Carvalho *et al.* demonstrated that patients with thyroid hormone β (THβ) resistance presented a severe deregulation of the hypothalamic-pituitary-thyroid (HPT) axis as well as abnormally elevated THs levels (Ortiga-Carvalho et al. 2014). On the contrary, patients with thyroid hormone α resistance showed nearly normal THs and TSH levels, implying a lower impact of the condition on the HPT axis. These results suggest that TRβ isoforms might play a crucial role in maintaining circulating THs levels by participating in the negative feedback of the HPT axis, an important target of EDCs (Ortiga-Carvalho et al. 2014). As a response to THs stimulation, TRs enter the nucleus and bind to thyroid hormone response elements (TREs), triggering the transcription of T3 target genes (Boas et al. 2012; Kim and Cheng 2013; Sandler et al. 2004). TRs can either act as homodimers or form heterodimers with retinoid X receptor (RXR) able to bind to TRE, inducing the stimulation or inhibition of associated genes (Hsu et al. 1995; Zhang et al. 1992). The thyroid system plays a central role in proper fetal development as well as preserving homeostasis during life, therefore maintaining a normal thyroid function and hormone levels is essential.

In recent years a link has been found between disturbed thyroid hormones' functions and exposure of man and wildlife to pollutants with endocrine disrupting properties (Ortiga-Carvalho et al. 2014). Although multiple sites of the thyroid system can be disrupted, it is extremely complex to assess them all as there is currently no practical battery that can cover all of these potential targets of EDCs. In the present paper, we designed a condensed thyroid hormone disruption panel aiming to evaluate both the potential agonistic and antagonistic aspects of chemicals towards TRβ, as well as their possible interferences with T4/TTR-binding. First, we developed the *in vitro* TRβ CALUX reporter gene assay based on the human U2OS cell line stably transfected to endogenously express TRβ. The cells were additionally transfected with a reporter construct containing a luciferase gene linked to two copies of TREs. In this bioassay, a TRβ activation stimulated by ligand-binding consecutively induces both the transcription of THs target genes and of gene coding for the luciferase protein. This method allows assessment of *in vitro* hormonal activity of agonistic chemicals by luciferase production quantification. Moreover, the TRβ CALUX assay can also be used to assess the activity of antagonistic compounds through cells pre-stimulation with the natural ligand T3. The current paper describes the design and operating mode of the new TRβ CALUX bioassay. In addition, this manuscript details the evaluation process used to assess the overall performance of both

agonistic and antagonistic methods. As no prior set of recommended chemicals was available at the time of the beginning of the study, we generated our own list of compounds selected on their thyroid-related activities reported in literature.

While a limited number of chemicals are known to directly interfere with TRβ, TTR is a well-known target for phenolic organo-halogen EDCs which may lead to thyroid depletion of the fetal brain and associated disorders (Cheek et al. 1999; Lans et al. 1993; Meerts et al. 2000; Montaño et al. 2012). In this manner, and as a complement to the TRβ CALUX bioassay, we included a TTR-binding assay to our method in order to assess TTR/THs potential interferences by EDCs. The TTR-binding assay involves the incubation of possible T4 competitor with a fixed amount of T4 and TTR. After separation from free remaining T4/compound, the fraction of T4 which bound to TTR can be measured using TRβ CALUX bioassay. This publication presents the detailed procedure to perform the optimized TTR-binding assay using TRβ CALUX as read-out system, hence referred as TTR-TRβ CALUX. The overall combined method's performance was evaluated by the analysis of known T4 competitor for TTR binding. In this report, we demonstrate that the newly developed TRβ CALUX method can be used as a readout of the existing TTR-binding assay, so as to assess potency of compounds for competing with T4 for TTR binding sites. Step by step, we detail the development of the TRβ CALUX and the TTR-TRβ CALUX bioassays as well as the intra-laboratory evaluation strategy used for each method (Fig 1.). We completed this study by investigating the applicability of the TTR-TRβ CALUX bioassay through a short case-study. We analyzed waste- and HPLC-water samples on the combined assay and highlighted net T4 competitive activities for TTR binding. This result showed a potential field application for the TTR-TRβ CALUX bioassay. Overall, three different CALUX-based bioassays (TRβ CALUX, anti- TRβ CALUX and TTR-TRβ CALUX) were developed and controlled for variance and reproducibility. This study shows that chemicals presenting both agonistic and antagonistic activities on the TRβ as well as T4 competitive properties for the thyroid transporter TTR can be accurately and efficiently predicted using our thyroid in vitro human testing panel.

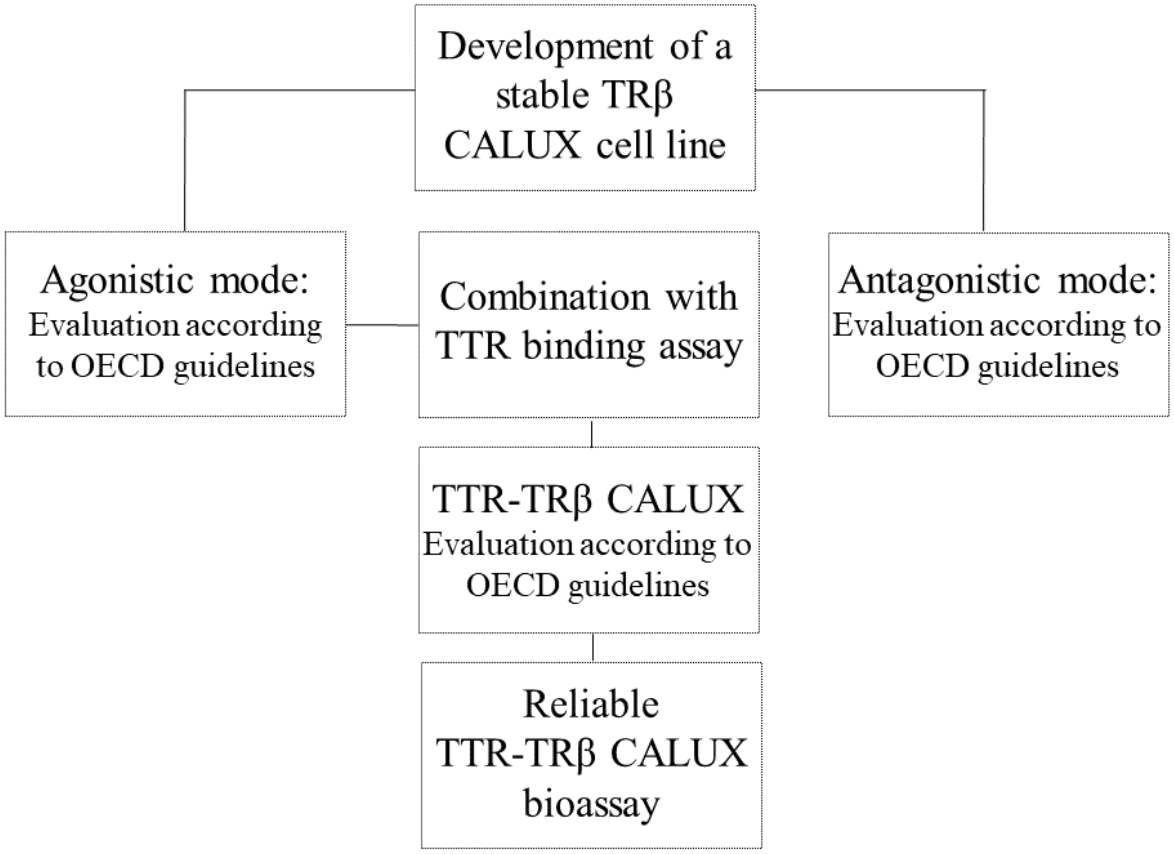

Figure 1. Study design for the evaluation of TRβ CALUX and TTR-TRβ CALUX bioassays. OECD: Organisation for Economic Co-operation and Development.

Materials and methods

TRβ CALUX reporter gene assay

The TRβ CALUX cell line is a human osteoblastic osteosarcoma U2OS line (American Type Culture Collection) stably transfected with a pSG5-neo-hTRβ plasmid to express TRβ receptors. The plasmid contains a full-length receptor and is expected to be able to heterodimerize with RXR expressed endogenously. The cells were additionally transfected with a pGL3 (Promega)-based reporter construct containing a luciferase gene linked to two copies of TREs (pGL3-2xTRE-Luc) (5'agctt/gatatcaggtcatttcaggtcagcatgc/gagctt/gatatcaggtcatttcaggtcagcatgc/g3' x2). The TRβ CALUX bioassay assesses TREs based responses triggered by TRβ activation, or RXR when present in a heterodimer. Either way the thyroid system will be affected: a TRβ/RXR activation will induce the transcription of the TRE following the same pathway than a TRβ homo-dimerization. This activation will consecutively induce the transcription of the T3-related genes and genes coding for luciferase protein.

TRβ CALUX were cultured as described before [2]. In brief, TRβ CALUX cells were cultured in Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) (Gibco, product no.: 31331-028) supplemented with 7.5% charcoal-stripped fetal calf serum (FCS), non-essential amino acids (NEAA) (Gibco, product no.: 11140-03) and 10U/mL penicillin and 10µg/mL streptomycin (P/S) (culture medium). Cells were maintained at 37°C and 5% CO_2 at all times and sub-cultured every 3-4 days.

One day prior to the exposure, cultured TRβ CALUX cells were trypsinized and re-suspended at a final concentration of $1x10^5$ cells/mL in DMEM/F12 medium without phenol red indicator supplemented with P/S, NEAA and 5% charcoal-stripped fetal calf serum (DCC) (assay medium). Cell suspension was seeded in clear 96-well plates and incubated for a minimum of 16 hours and maximum 24 hours in a humidified atmosphere at 37°C and 5% CO_2. The next day, the medium was removed, and cells were incubated with 200µL of exposure medium consisting of DMEM/F12 supplemented with P/S, NEAA and the compound to be assessed dissolved in DMSO. To avoid any aspecific proteins' interference, the exposure medium does not contain DCC. For each chemical, 8 serial dilutions were tested to a final concentration of 0.1% DMSO, in exposure medium. Each dilution of the test chemicals, including the reference compounds, were tested in triplicate. Plates were placed back in the incubator at 37°C and 5% CO_2 for a period of 24±2 hours. After incubation, the medium was discarded, and cells were lysed using 30µL/well of a Triton-lysis buffer. Plates were shaken for 10 minutes after which luciferase activity in cellular lysates was measured for 4 seconds using

a luminometer (Tristar, Berthold). The described procedure is used to determine the agonistic potency of chemicals, reflected by light production which can be quantified. For antagonistic assessment, the same procedure was followed with the exception that the exposure medium was supplemented with a non-saturating level of T3 (1.7E-6 M). Therefore, an antagonistic compound is expected to compete with T3 for TRβ binding resulting in a reduction in light emission.

For all measurements, a full dose response dilution series of the reference compound was included on each plate for response quantification. To strengthen quality control, a positive and a negative compound control as well as a solvent control (DMSO) were added to each plate. Regarding the agonistic mode of the TRβ CALUX bioassay, the selected reference compound was the natural ligand T3. 3,3'5-triiodothyroacetic acid (TRIAC), a T4-like compound was chosen as positive control, and bisphenol A (BPA), a TRs antagonist, as negative control. For the antagonist approach, deoxynivalenol was set as the reference, T-2 mycotoxin (T2-toxin) as the positive control and amiodarone as a negative control.

TTR- TRβ CALUX: the competitive binding assay and analysis

Prior to the TTR-binding assay, all compounds including the reference tetrabromobisphenol A (TBBPA) were dissolved in DMSO in dark glass vials. TTR and T4 working solutions were prepared in Tris buffer, made by mixing 12.11g of Tris base (CAS no.: 77-86-1, Sigma Aldrich), 5.84g NaCl (CAS no.: 7647-14-5, Sigma Aldrich) and 0.372g of ethylenediaminetetraacetic acid (EDTA) (CAS no.: 60-00-4, Sigma Aldrich) in 1L of HPLC water (J.T Baker). The final Tris buffer pH was adjusted to 8.0. Expected T4 and TTR working solution concentrations were 0.08µM and 0.190µM, respectively. The TTR-binding assay is based on an incubation mixture containing 100µL of T4, 50µL TTR and 5µL of tested or model competitor compound, prepared in a 1.5mL Eppendorf tube (Fig. 2). In order to prevent TTR denaturation, it is important to handle the protein carefully and avoid mixing with a vortex at any point of the procedure. After one hour of incubation at room temperature, tubes were stored at 4°C for at least 20 minutes to overnight. As a next step, self-made Bio-Gel P-6DG columns were prepared to separate T4- or test compound-TTR complexes from unbound compounds. In short, columns were prepared in 1ml disposable Omnifix®-F Solo syringes (B. Braun, product no.: 9161406V). For one 96-well plate, 3g of Bio-Gel P-6DC (Bio-Rad, product no.: 150-0739) was dissolved in 30mL of Tris buffer and incubated at 4°C overnight. From this point, tubes containing mixtures were kept on ice at any time to avoid the dissociation of the compound-TTR complexes. Syringes were placed into 15mL Greiner tubes and approximately 1.3mL of Bio-Gel was added, followed by a minute of centrifugation at 120g at room temperature. Bio-Gel P-6DG columns were transferred into clean collector 15mL Greiner tubes

placed on ice, whereupon incubate mixtures were immediately loaded. Columns were centrifuged for an additional one minute at 120g. Collected fractions can either be stored up to 8 days at room temperature or directly tested in the TRβ CALUX bioassay.

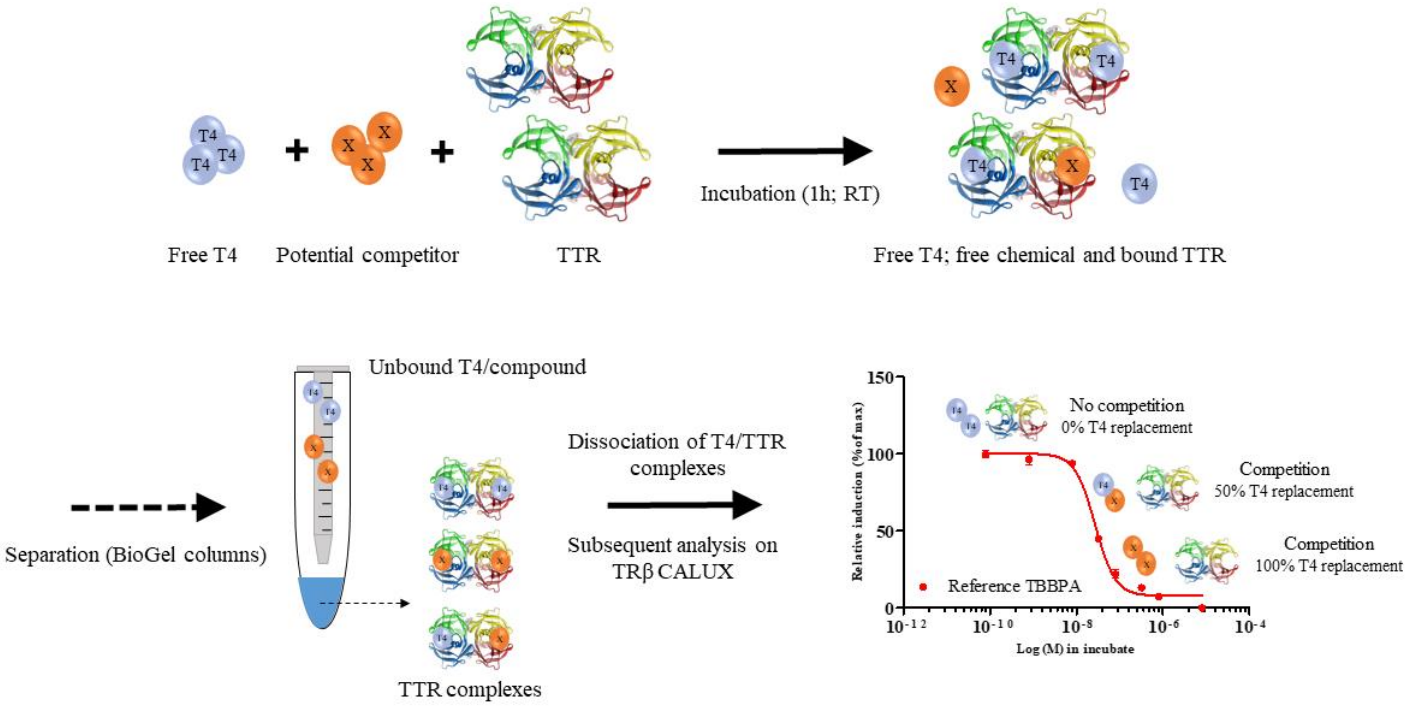

Figure 2. Diagram of the TTR-TRβ CALUX bioassay. Free T4, potential competitor chemical and TTR are incubated as a mixture for 1h at room temperature. Unbound T4 and free chemical molecules are separated from TTR complexes using BioGel columns. The bound fraction containing T4/TTR complexes is left at room temperature for few minutes in order to allow T4 to dissociate prior to analysis on the TRβ CALUX bioassay.

The method of TRβ CALUX testing was carried out essentially as described in 2.1 with only slight variations in the exposure method. In short, exposure medium was prepared by adding 140µL of TTR-T4 mixture to 500µL of phenol red-free DMEM/F12 medium supplemented with non-essential amino acids (NEAA) and P/S. Additionally, quality control was included to each plate by filling one triplicate with 200µL of 8e-6M T4 at a final DMSO concentration of 0.1%. All data points were carried out in triplicate. After 24±2-hour incubation, the exposure medium was removed, and cells were lysed using 10µL/well triton-lysis buffer. Afterwards, the luciferase activity in cellular lysates was measured with a luminometer (Berthold) as described above.

Each TTR-binding assay procedure included the solvent DMSO, in which the reference compound, competing chemicals and sample extracts were dissolved, as a solvent control. For

normal testing, one dilution series of the reference compound TBBPA was added to each plate as a quality control for the proper functioning of the assay. For each compound, a second negative control including TTR and the assessed competitor, without the addition of T4, was included to the procedure. This step allowed to evaluate potential direct interactions between the tested compound and TRβ.

Compound selection and evaluation guidelines

The evaluation of the bioassays was conducted following the Organisation for Economic Co-operation and Development (OECD) guidelines (OECD 2015). According to OECD requirements intra- and inter-laboratory studies have to be performed. In this paper, only the intra-laboratory phase was conducted. As in the foreseeable future, TRβ CALUX bioassays will be evaluated side by side with other thyroid-relevant endpoints such as thyroperoxidase (TPO), TBG and deiodinase activities as a part of a larger study organized by the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM). The intra-laboratory evaluation described in this paper required the generation of three lists of compounds chosen for their properties to interact or not with the TRβ and/or TTR. While the selection was mainly built on literature review, we selected few additional chemicals from our own internal database. For intra-laboratory assessment of the TRβ CALUX bioassay (agonistic mode), an initial set of eighteen chemicals were chosen (See Annex 2, Table 1). There are very few compounds referred in literature for their agonistic interaction with TRβ besides the four well known TRb agonists (T3, T4, 3,3'5,5'-tetraidothyroacetic acid TETRAC and TRIAC). Hence, along with these chemicals, we decided to also include two compounds from our internal screening that showed a weak response on the TRβ (2-acetamidofluorene 2-AAF and all trans-retinoic acid atRA). To complete this set we incorporated 10 non-active chemicals, 5 were derived from a literature review (1-850, amiodarone, bisphenol A (BPA), endosulfan and TBBPA) and 5 from our own database (aflatoxin B1, dieldrin, methoxyacetic acid, valproic acid and vinclozolin). T2-toxin and hydroquinone were also analyzed on the TRβ CALUX bioassay but not included in the performance calculations as no prior data were reported at the time of the study.

A list of fifteen chemicals were established to test the reliability of the antagonistic TRb CALUX assay (See Annex 2, Table 2). Nine antagonistic compounds were selected from literature (1-850, 4-nonyphenol, BPA, deoxynivalenol, dibutylphthalate, endosulfan, pinoresinol, TBBPA and zearalenone). We also included the mycotoxin T2-toxin due to its antagonistic characteristics found in previous unpublished results obtained in our laboratory. Four chemicals were chosen for their non-active properties (aflatoxin B1, amiodarone,

dronedarone and T3). We decided to include the RXR ligand at-RA to our study to assess its potential activity on the TRβ.

To test the TTR-TRβ CALUX bioassay, eleven compounds were assessed for their interference with the T4-TTR binding. Nine compounds were selected based on published information on their capacities to interact with either TRβ or TTR (4-nonyphenol, BPA, diethylstilbestrol (DES), pentabromophenol (PBP), pentachlorophenol (PCP), perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), TBBPA and tetrachlorobisphenol A (TCBPA)). Alachlor is known to be inactive and was chosen from literature. T2-toxin was included based on results obtained during an internal screening.

The evaluation procedure included a pre-screen step followed by at least two independent comprehensive measurements (biological replicate = 3). The general objectives of a pre-screen are to determine the solubility of test chemicals in both DMSO and assay medium and assess their potential cytotoxicity towards U2OS cells. Therefore, during this pilot phase, all concentration tested in the (anti-)TRβ CALUX bioassays were also assessed on the Cytotox CALUX® bioassay that can be used to assess non-specific CALUX assay interferences, e.g. caused by cytotoxicity (Sonneveld et al. 2005; Van der Linden et al. 2014). In short, the Cytotox CALUX bioassay uses U2OS cell line which constitutively express luciferase, the addition of cytotoxic compound to the cells would consequently result in a reduction in light production quantifiable with a luminometer. For the pre-screen, 8 serial dilutions of each compound were made from the highest soluble concentration using 10-step dilutions. In order to obtain a proper full dose response curve, the highest concentration tested should not be cytotoxic (cytotoxicity ≤ 20%). If one or several dilutions induced cytotoxicity, the test was repeated using lower concentrations (Annex 1. Table 1-3). Results of the pre-screen study were used to refine the concentration ranges of the test compounds to be used for the comprehensive studies. Unlike the pre-screen phase, comprehensive measurements were based on 3-step dilutions prepared from the highest concentration of each chemical not showing cytotoxicity. In case the results of the comprehensive tests were divergent to each other, a third measurement was added to the study increasing the biological replicates from 3 to 4. Each dilution of the test chemicals including the reference compounds were tested in triplicate on the same 96-well plate.

Data handling and acceptance criteria

Luciferase activity per well was expressed as Relative Light Units (RLUs) and corrected for background using solvent control measurements (pure DMSO). The maximum signal response elicited by the reference compound was set to 100% (full receptor activation).

Subsequently, all registered RLU produced by the test compounds or samples were expressed in % of maximum response of the reference compound. The statistical software package GraphPad Prism 5.0 was used to fit transformed data (non-linear regression, variable slope, 4 parameters and robust fit). Compounds were scored positive if a minimum of two consecutive concentrations showed an increase of at least 10% (PC_{10}, agonist mode) or a decrease in TRβ activation of more than 20% (PC_{80}, antagonist mode) of the maximal effect of the reference compound. Depending on the selected mode of the TRβ CALUX bioassay, either EC_{50} or IC_{50} were evaluated using the same software package. EC_{50} refers to the maximal induced effect of the compound tested. For the antagonist procedure, 50% maximal inhibitory concentration (IC_{50}) was evaluated for each chemical and compared with available data in literature, if available. EC_{50} and IC_{50} can be used to assess and rank the potencies of tested chemicals. For agonistic studies, the limit of detection (LOD; average (solvent blank) +3*SD (solvent blank))) and the limit of quantitation (LOQ; average (solvent blank) +10*SD (solvent blank))) were also determined for all plates based on the standard deviation of the solvent blank DMSO. Z-factor was systematically calculated for the reference compound according to the following formula (1). Z-factor values should be above 0.6 to fit the acceptance criteria.

(1) $$\text{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}.$$

For the evaluation of intra-laboratory assessment results, the coefficient of variance (%CV) and reproducibility ($\%VC_R$) were calculated. The intra-laboratory coefficient of variance was determined based on the average log (EC_{50}) and log (PC_{10}) (agonist), and log (IC_{50}) and log (PC_{80}) (antagonist) derived from both comprehensive test runs. In addition, the intra-laboratory reproducibility variation coefficient was determined based on the log (EC_{50}) and log (PC_{10}) (agonist), and log (IC_{50}) and log (PC_{80}) (antagonist) derived from both comprehensive test runs. The reproducibility is the variation in measurements taken by a single/multiple persons or instruments on the same item at different time-points. Reproducibility was calculated according to formula (2).

(2) $$\%VC_R = \sqrt{\frac{\sum_{i=1}^{n}\left(\frac{x_{i1}-x_{i2}}{0.5(x_{i1}-x_{i2})}\right)^2}{2n}} \times 100\%$$

$\%VC_R$ = reproducibility variation coefficient

x_{i1} = measuring result i^{th} determination first observation

x_{i2} = measuring result i^{th} determination second observation

n = number of determinations

The qualitative intra-laboratory concordance of classifications (active/non-active) was determined based on the results of two independent comprehensive tests under reproducibility conditions. From these results, overall accuracy, sensitivity, and predictability of the methods were calculated and expressed as a percentage.

Demonstration study using water samples

Spiked water sample

In order to assess the TTR-TRβ CALUX assay applicability for environmental studies, HPLC-grade water was spiked with three pure compounds: DES, TCBPA and PBP, under controlled conditions. These three chemicals were defined as T4-competitors in the previous TTR-TRβ CALUX evaluation, allowing us to further calculate the relative potency resulting from their mixture. All compounds were dissolved in methanol (MeOH) to a stock concentration of $2.5E^{-3}$M. 120µL of this mixture was added to 250mL HPLC-grade water, resulting in a final concentration of $4.1E^{-7}$M for each of the spike-compounds. A procedure blank, consisting of 250mL of water with MeOH, was also included into the experiment. Samples were extracted using Solid Phase Extraction (SPE) columns (OASIS HLB SPE cartridges 500mg 6 cc, Waters product no.: 186000115) conditioned with approximately 500ml of water and eluted with 10ml of MeOH followed by 10ml of acetonitrile. Fractions were pooled and evaporated until dryness under a gentle stream of nitrogen at 40°C. The final extracts were dissolved in 100µL of DMSO in order to keep the concentration of the spiking mixture and extract equal. The diluted extracts (dilution series 1-3-10-30-100-300-1000-3000-10000-30000x) as well as the neat spiking mixture were tested on the TTR-TRβ CALUX assay and analyzed following the procedure previously described in section 2.2.

Environmental water samples

Two effluent samples collected from a wastewater treatment plant and one HPLC-water blank were extracted and analyzed following the previously described method (2.5.1). In short, 1L of each sample were extracted through SPE columns eluted with 10ml of MeOH and 10ml of acetonitrile. After evaporation, the final extracts were dissolved in 150µl of DMSO and used to prepare serial dilution (1-3-10-30-100x). A first range finding analysis was performed in the TTR-TRβ CALUX bioassay to obtain the appropriate dose-response range of the sample extracts. Based on which the dilution series was adjusted (1-1.5-2-2.5-3-10x) and extracts were analyzed in the TTR-TRβ CALUX bioassay.

Results

Determination of thyroid receptor agonistic activity of compounds using TRβ CALUX bioassay

The sensitivity and responsiveness of the TRβ CALUX assay were assessed by measuring the signal of the reference compound T3 along with eighteen chemicals (see Annex 2. Table 1.). This set contained known TRβ agonists and chemicals that showed no affinity towards the TRβ receptor, also referred to as "non-responders". As a start, the performance and stability of the agonist test were determined by comparing EC_{50} values of the reference compound, T3 from the different measurements (Table 1; Fig. 3). The range in EC_{50} values was small, reflected by a %CV of 0.8%. Additionally, the LOD and LOQ were calculated for each measurement and compared. The variations in LOD and LOQ values were low (%CV≤1%) clearly indicating that the TRβ CALUX bioassay is a stable bioassay producing repeatable results.

Table 1. Log EC_{50}, PC_{10}, PC_{50}, LOD and LOQ values for the reference agonistic compound T3 on the TRβ CALUX bioassay.

Run	log EC_{50} (M)	log PC_{10} (M)	log PC_{50} (M)	log LOD (M)	log LOQ (M)
Pre-screen	-9.77	-10.28	-9.74	-10.99	-10.51
Comprehensive 1	-9.88	-10.37	-9.85	-10.84	-10.36
Comprehensive 2	-9.92	-10.43	-9.92	-10.80	-10.32
Average	-9.86	-10.36	-9.83	-10.88	-10.40
%CV	0.8%	0.7%	0.9%	0.9%	1.0%

Note: Average represents the average values and %CV corresponds to the calculated coefficient of variation for three measurements.

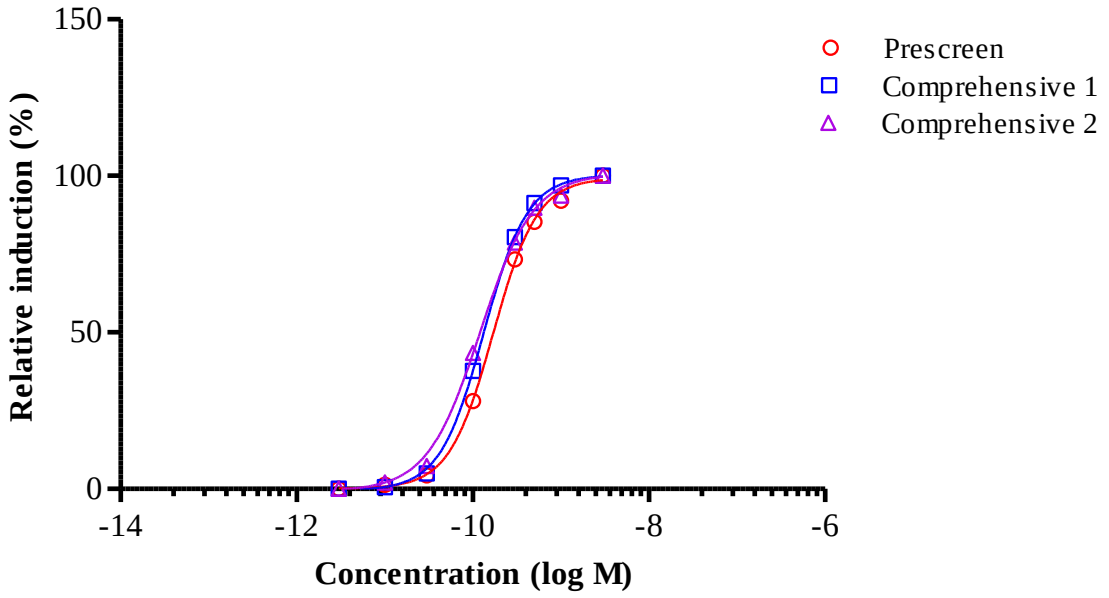

Figure 3. Dose-response curves obtained with the reference compound T3, analyzed at least twice for the prescreen and each comprehensive. Each point represents the average of these measurements.

Next, the panel of test compounds was tested on the TRβ CALUX assay by two different technicians. Out of these eighteen chemicals, six scored positive on the agonistic bioassay (Table 2). The TRβ CALUX bioassay gave a general z-factor of 0.89. Whereas T3, T4, TRIAC and TETRAC provided similar full dose-response curves, both at-RA and 2-AAF showed differences in shape and lower activity resulting in an absence of PC_{50} value (Fig. 4). All the natural THs, as well as their analogues TRIAC and TETRAC (T3 and T4-like, respectively), showed agonistic activities. Regarding this valuation, 2-AAF scored positive due to the fact of its low but still noticeable activity (Fig 4). Indeed, this compound showed a slight partial agonistic response on the TRβ CALUX bioassay matching preset OECD scoring criteria of at least two consecutive points above 10% T3's activity to be considered as an active chemical. Following the same criteria, at-RA was also scored positive in the bioassay. Whereas 2-AAF showed no cytotoxicity at high concentration, at-RA was reported as cytotoxic at the highest concentration tested (i.e. 3.E-5M and higher) (data not shown). Although the activity was quite low both 2-AAF and at-RA were defined as activators of the TRβ. From the initial list, twelve compounds (1-850, aflatoxin B1, amiodarone, methoxyacetic acid, BPA, dieldrin, endosulfan, hydroquinone, T2-toxin, TBBPA, valproic acid and vinclozolin) scored negative on the agonist TRβ CALUX bioassay (data not shown).

Table 2. Log EC_{50}, PC_{10} and PC_{50} values for positive expected and tested compounds in agonist TRβ CALUX bioassay.

		log EC_{50} (M)	log PC_{10} (M)	log PC_{50} (M)	Relative potency (PC_{10})	Measured activity	Reported activity
T3	**avg**	-9.44	-10.13	-9.48	1.0	active	active
	%CV	0.80%	1.60%	0.80%			
2-AAF	**avg**	-6.15	-5.54	n.a.	2.8	active	unknown
	%CV	0.1%	3.6%	n.a.			
at-RA	**avg**	-6.26	-6.25	n.a.	5.9E-1	active	unknown
	%CV	0.3%	0.4%	n.a.			
T4	**avg**	-9.01	-9.75	-9.05	5.8	active	active
	%CV	0.4%	0.6%	0.1%			
TRIAC	**avg**	-10.8	-11.6	-10.9	3.5E-1	active	active
	%CV	0.5%	0.7%	0.3%			
TETRAC	**avg**	-9.30	-9.73	-9.02	2.2E+1	active	active
	%CV	0.3%	1.2%	3.7%			

Note: Average represents the average values and %CV corresponds to the calculated coefficient of variation for two comprehensive measurements.

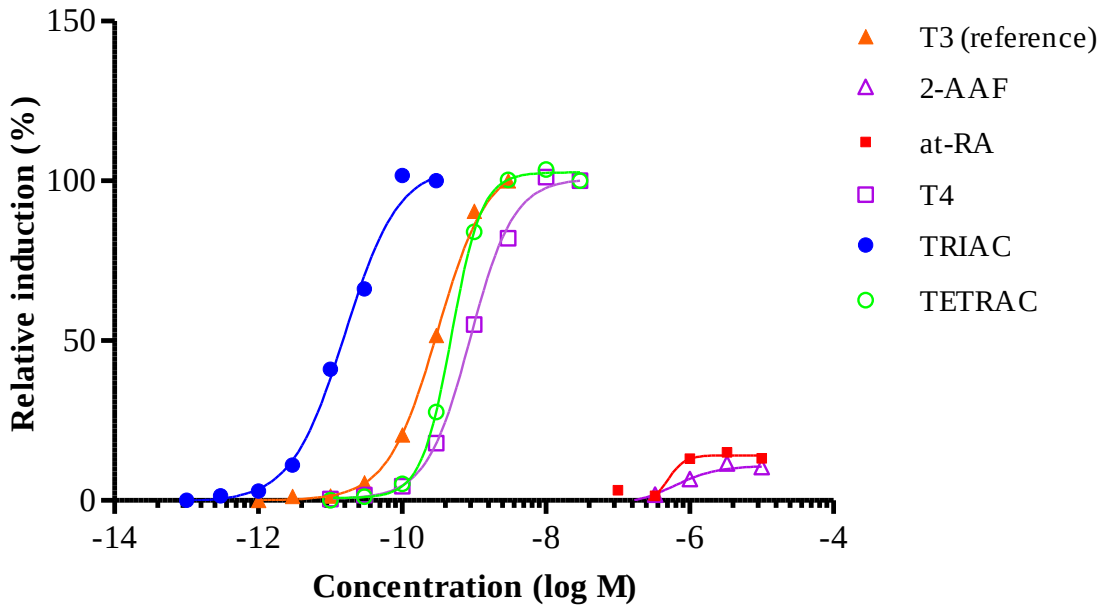

Figure 4. Dose-response curves of positive compounds obtained in the agonist mode of the TRβ CALUX bioassay.

Determination of thyroid receptor antagonistic activity of pure compounds using TRβ CALUX bioassay

The accuracy and reliability of the antagonistic mode of the TRβ CALUX bioassay were investigated using the reference compound deoxynivalenol along with 15 pure chemicals (see Annex 2. Table 2.). Deoxynivalenol was first assessed alone in three separate experiments performed by at least two technicians. Both prescreen and comprehensive tests gave similar dose-response curves, resulting in low variation between the calculated log IC_{50} (%CV = 1.4%) (Table 3. Fig. 5). The repeatability of the method was reflected by an overall %CV ≤ 4.1% while the calculated reproducibility $\%VC_R$ was even lower (=1.35%). These finding confirm the stability of the antagonistic TRβ CALUX bioassay in generating reproducible results.

Table 3. Log IC_{50}, PC_{50}, PC_{80} values for deoxynivalenol in the antagonist mode of the TRβ CALUX bioassay.

Run	log IC_{50} (M)	log PC_{50} (M)	log PC_{80} (M)
Pre-screen	-6.28	-6.29	-6.68
Comprehensive 1	-6.14	-6.15	-6.58
Comprehensive 2	-6.14	-6.18	-6.18
Average	-6.18	-6.21	-6.48
%CV	1.3%	1.2%	4.1%

Note: Average represents the average values and %CV corresponds to the calculated coefficient of variation for three measurements.

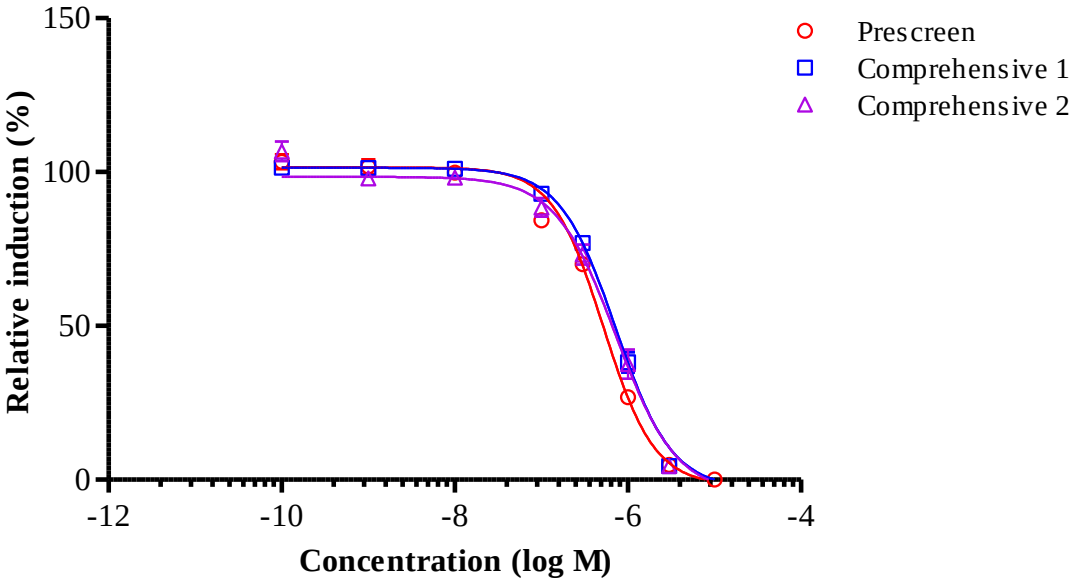

Figure 5. Dose-response curves of deoxynivalenol obtained in the antagonist mode of the TRβ CALUX bioassay. The reference compound was analyzed at least twice for the prescreen and each comprehensive. Each point represents the average of these measurements.

Fifteen pre-selected chemicals were tested in the TRβ CALUX bioassay in order to determine their thyroid receptor antagonistic potential. Nine chemicals showed antagonistic activity with different potency and shape of dose-response curves (Table 4, Fig. 6.a.b.). None of the tested compounds were found to be cytotoxic in the assessed concentration range indicating that the decrease in activity represents antagonistic activity. 1-850, at-RA, amiodarone, BPA and zearalenone all scored positive but did not give a full-dose response curve as their higher concentration did not induce full antagonism. Alongside with 4-nonyphenol and deoxynivalenol, TBBPA showed a full dose-response curve indicating the complete inhibition of the TRβ receptor activation. The mycotoxin T2 showed activity in the anti-TRβ CALUX bioassay which was higher in $\log IC_{50}$ than any in the compound set. Six compounds (aflatoxin B1, dibutylphthalate, dronedarone, endosulfan, pinoresinol and T3) did not show activity nor fit the preset criteria of the bioassay.

As a quality criterion, %CV was calculated for each compound and an average z-factor was given (0.72). All chemicals showed good repeatability in their results and thereby showed a good stability of the antagonistic TRβ CALUX bioassay ($0 \leq \%CV \leq 3.5\%$).

Table 4. Log IC_{50}, PC_{80} and PC_{50} values for positive expected and tested compounds in the antagonist mode of the TRβ CALUX bioassay.

		Log IC_{50} (M)	Log PC_{80} (M)	Log PC_{50} (M)	Relative potency (PC_{80})	Measured activity	Reported activity
deoxynivalenol	**avg**	-6.22	-6.54	-6.21	1	active	active
	%CV	0.60%	0.70%	3.50%			
1-850	**avg**	-5.02	-5.19	-4.93	4.50E-02	active	active
	%CV	1.00%	0.20%	0.30%			
4-nonylphenol	**avg**	-5.45	-5.63	-5.45	1.20E-01	active	active
	%CV	1.00%	2.70%	1.60%			
at-RA	**avg**	-4.75	-4.96	-4.75	2.60E-02	active	unknown
	%CV	1.10%	0.20%	3.50%			
amiodarone	**avg**	-5.59	-5.95	-5.59	2.60E-01	active	active
	%CV	2.30%	0.70%	3.50%			
BPA	**avg**	-4.96	-4.73	-4.45	1.50E-02	active	active
	%CV	0.50%	1.70%	0.00%			
TBBPA	**avg**	-6.07	-6.62	-6.15	1.2	active	active
	%CV	1.00%	0.70%	3.50%			
zearalenone	**avg**	-4.51	-4.8	-4.51	1.80E-02	active	active
	%CV	0.30%	1.10%	0.40%			
T2-toxin	**avg**	-8.47	-10.38	-10.5	6.90E+03	active	unknown
	%CV	0.00%	0.10%	0.00%			

Note: Avg represents the average and %CV corresponds to coefficient of variation for two comprehensives.

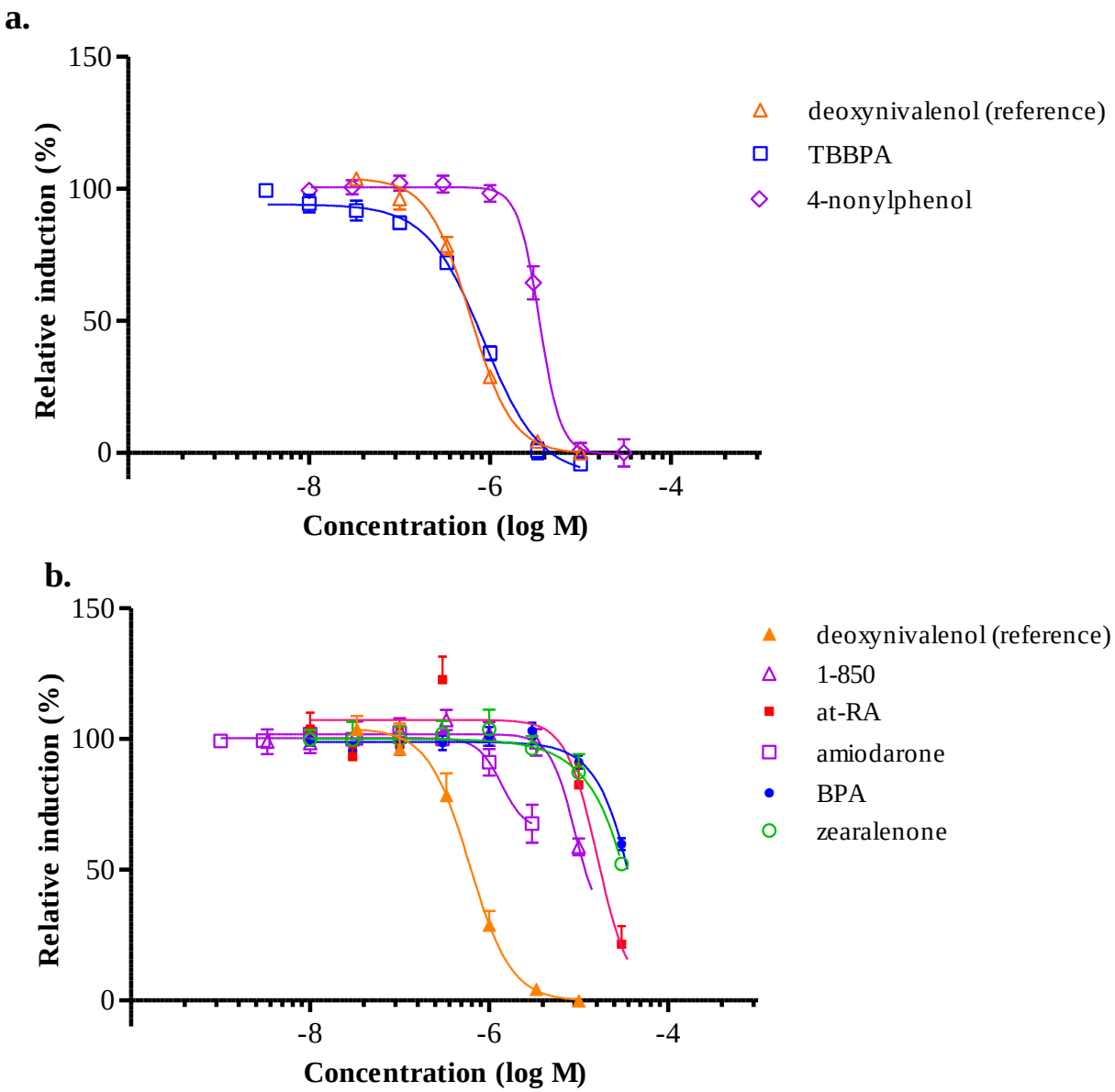

Figure 6.a.b. Representative dose-responsive curves of tested chemicals obtained using the antagonist mode of the TRβ CALUX bioassay. Each data point is the mean of at least two measurements.

Evaluation of the TTR-TRβ CALUX bioassay

The test design of the evaluation of the TTR-TRβ CALUX bioassay was based on eleven pure compounds selected on their abilities to interact with TTR according to the literature (see Annex 2. Table 3). As a start, the performance of the reference compound TBBPA was investigated in three independent experiments performed by at least two different technicians. The shape of the dose-response curves was similar between the individual experiments, giving comparable log IC_{50} and PC_{80} (-7.60M±0.10 and -8.01M±0.9, respectively) (Table 5; Fig. 7.).

These results indicate the stability of the TTR bioassay when used in combination with the TRβ CALUX.

Table 5. Log IC_{50}, PC_{50} and PC_{80} values for TBBPA reference compound measured in the TTR-TRβ CALUX bioassay.

Run	log IC_{50} (M)	log PC_{50} (M)	log PC_{80} (M)
Pre-screen	-7.66	-7.66	-8.10
Comprehensive 1	-7.77	-7.64	-7.92
Comprehensive 2	-7.57	-7.59	-8.0
Average	-7.67	-7.63	-8.01
%CV	1.3%	0.5%	1.1%

Note: Average represents the average values and %CV corresponds to the calculated coefficient of variation for three measurements.

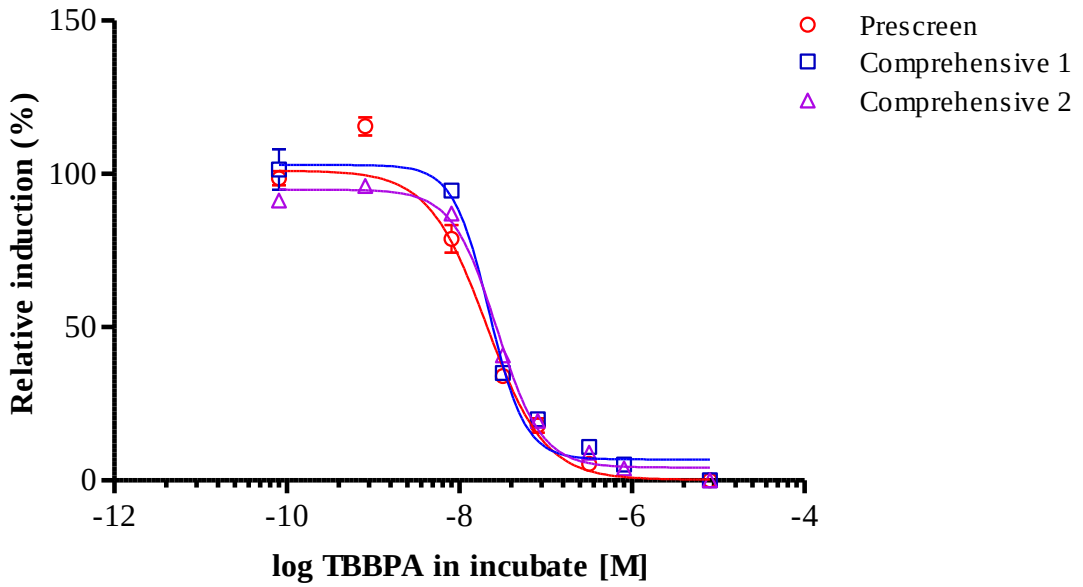

Figure 7. Dose-response curves obtained with the reference compound TBBPA in TTR-TRβ CALUX. TBBPA was analyzed at least twice for the prescreen and each comprehensive. Each point represents the average of these measurements.

In addition, a panel of potential T4-TTR binding competition positive chemicals was assessed following the same procedure. Associated TTR-binding potencies were expressed as log concentration at 50% inhibition for T4 (Table 6). A general z-factor value of 0.74 was calculated. For each compound, %CV were calculated for logIC_{50}, logPC_{80} and logPC_{50}. All test chemicals (4-nonyphenol, BPA, DES, PBP, PCP, PFOA, PFOS, T2-toxin, TBBPA and TCBPA) with the exception of alachlor, showed binding inhibition responses and were reported as competitors for T4-TTR binding (Fig. 8). BPA demonstrated capacity to compete with T4 for

binding TTR, however no IC_{50} could be calculated due to the fact that this compound did not give a full dose-response curve (data not shown). Out of eleven compounds tested, ten showed T4-competing properties with a stronger effect from perfluorinated chemicals PFOA and PFOS and the reference chemical TBBPA.

Table 6. Log IC_{50}, PC_{80} and PC_{50} values for positive expected and tested compounds in TTR-TRβ CALUX bioassay.

		IC_{50}	PC_{80}	PC_{50}	Relative potency (PC_{80})	Measured activity	Reported activity
TBBPA	avg %CV	-7.81 0.50%	-8.06 0.30%	-7.79 0.10%	1	active	active
4-nonylphenol	avg %CV	-4.12 3.70%	-4.54 17.30%	-4.23 9.90%	3.00E-04	active	active
BPA	avg %CV		-4.27 5.60%	-3.32 5.10%	1.60E-04	active	active
DES	avg %CV	-6.68 1.50%	-7.03 2.00%	-6.67 0.70%	9.30E-02	active	active
PBP	avg %CV	-6.59 0.50%	-6.9 3.30%	-6.61 1.50%	6.90E-02	active	active
PCP	avg %CV	-4.47 0.30%	-4.79 1.30%	-4.47 0.20%	5.40E-04	active	active
PFOA	avg %CV	-7.5 1.30%	-7.74 1.10%	-7.41 2.20%	4.80E-01	active	active
PFOS	avg %CV	-7.22 0.80%	-7.45 0.50%	-7.15 0.40%	2.50E-01	active	active
T2-toxin	avg %CV	-6.0 0.50%	-6.08 1.20%	-5.91 0.50%	1.00E-02	active	unknown
TCBPA	avg %CV	-6.77 1.80%	-7.03 1.10%	-6.73 1.40%	9.30E-02	active	active

Note: Avg represents the average and %CV corresponds to coefficient of variation for two comprehensive measurements. For abbreviations refer to 2.4.

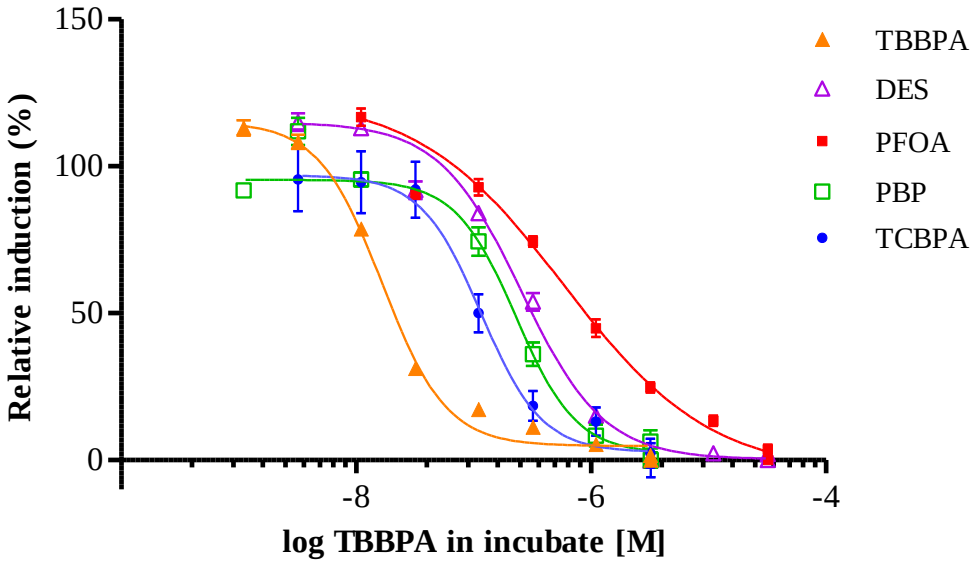

Figure 8. Dose-response curves of DES, PFOA, PBP, TBBPA and TBCPA in the TTR-TRβ CALUX. Each data point is the mean of at least two measurements.

Demonstration study: water samples

Spiked water sample

Water (HPLC-grade) was spiked with a mixture of three compounds (DES, TCBPA and PBP) with dilution steps of 1, 3, 10, 30, 100, 300, 1000, 3000, 10000 and 30000. After the extraction procedure, extract obtained from spiked water was tested in the TTR-TRβ bioassay alongside the original spiking mixture (Fig. 9). So as to evaluate the quality of the SPE extraction, results obtained from the mixture itself and the spiked water sample were compared revealing a recovery value of 101.3% based on PC_{80} values. The global potency of the mixture was calculated and expressed as TBBPA equivalent, giving a value of 3.17E-05M. Overall, this experiment suggested that the TTR-TRβ bioassay used in combination with SPE-based extraction might be suitable for water sample analysis.

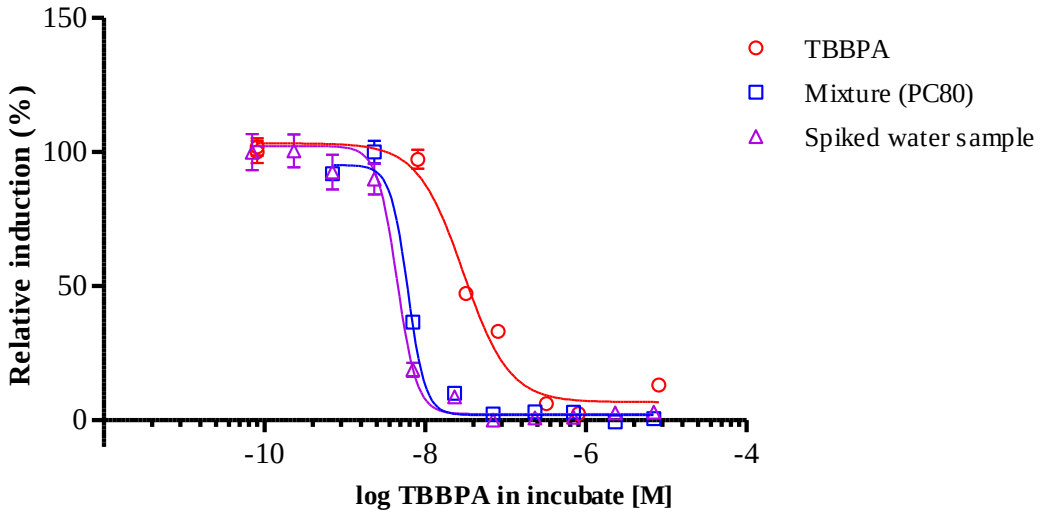

Figure 9. Dose-response curves from reference compound TBBPA, mixture and extract from spiked water sample tested in TTR-TRβ-CALUX. The mixture consisted of DES, PBP and TCBPA at a final individual concentration of 4.1e-7M.

Environmental water samples

To determine the applicability of the TTR-TRβ bioassay for analysis of potential thyroid disrupting chemicals in environmental waters, two wastewater treatment plant samples were analyzed. Along with these samples, HPLC-water was used as a procedure blank control and did not show any binding activity towards TTR. Both samples from the wastewater treatment plant demonstrated a significant dose-dependent T4-TTR binding competition (Fig. 10).

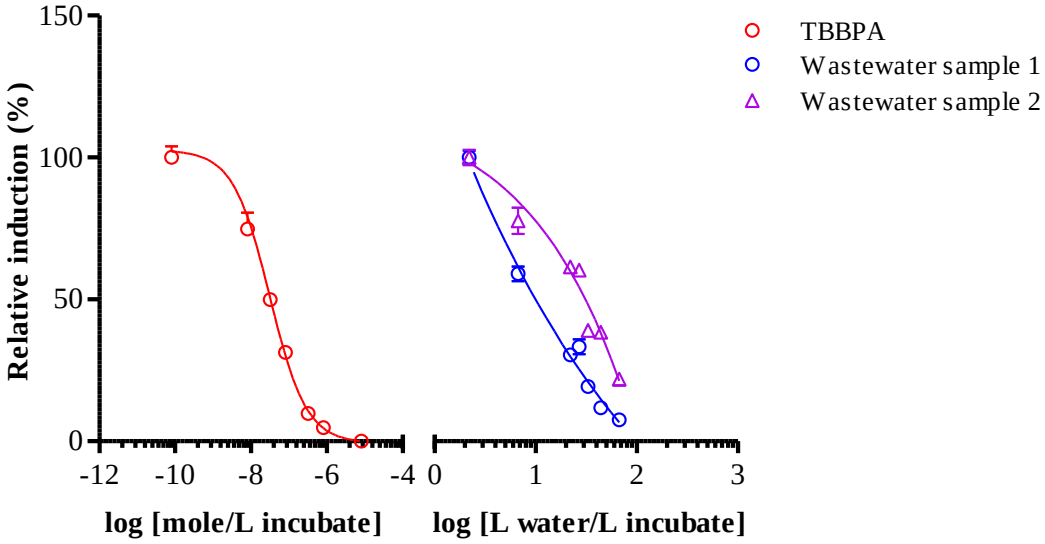

Figure 10. Dose-response relationships of wastewater and HPLC-water samples analyzed in TTR-TRβ CALUX bioassay.

Discussion

Regarding the fact that no set of recommended chemicals for thyroid disruption was available at the time the study began (2015), a preliminary review was performed so as to generate a list of reference compounds suitable for the evaluation of each aspect of the thyroid panel. From literature, nine, twelve and ten compounds were chosen for assessing the agonistic, antagonistic TRβ CALUX and TTR-TRβ CALUX assays. Along with these reference chemicals, nine, three and one other compounds were respectively added to the sets. These compounds were considered of interest based on results obtained during an internal screening. While jointly evaluated on the panel, none of these additional chemicals were used in predictability nor accuracy calculations, which were strictly based on the compounds properly reported in literature. The evaluation of each thyroid-related bioassay relies on this preliminary work.

In 2016, Wegner et al. reviewed Tier 1 endocrine screening assays and generated a list of 34 reference chemicals to be used to assess thyroid system's alterations, however it is unclear which mechanism were involved in this disruption. In this paper, we focused on assessing the (ant)agonistic potency of compounds on one TH receptor using a reporter gene assay (TRβ CALUX) and one non-receptor-based TH- response (TTR assay), the later one based on previous research of our group on competitive binding interference of compounds to TTR. Hence, it was crucial for our study to select compounds with available *in vitro* information regarding their potential receptor or transporter disrupting activities. Nevertheless, it would be of interest to analyze the set of chemicals provided by Wegner et al. (2016) in the future.

Within this study, we have developed a robust CALUX-based test method to assess the agonistic and antagonistic potency of chemicals towards the TRβ, as well as T4-TTR binding competition potencies of chemicals. The evaluation of both agonistic and antagonistic TRβ CALUX assays showed low variations in EC_{50} (≤0.8%) and IC_{50} (≤4.1%) measurements and high z-factor values (0.89 and 0.72, respectively), indicating a good reproducibility of the methods.

The TRβ CALUX assay generated consistent results in comparison with existing data. The rank order of agonistic potency of active THs (T3 and T4) and chemicals with similar structure (TETRAC and TRIAC) was established as TRIAC>T3>TETRAC>T4, matching the results found in literature (Cheek et al. 1999; Gutleb et al. 2005). Unlike these very active compounds ($logEC_{50}$≥9.00), at-RA and 2-AAF showed a weak activity in the TRβ CALUX bioassay ($logEC_{50}$=6.26 and 6.15, respectively). at-RA is a well-established ligand for retinoic X receptors (RXR), receptor able to act as heterodimer with TRβ (Hsu et al. 1995; Zhang et al.

1992). In our case, although a higher concentration of at-RA led to cytotoxicity (Annex 1. Table 1), we observed that this compound elicits an aberrant transactivation of the TRβ which may be the result of RXR binding rather than a direct interaction with TRβ itself. 2-AAF also showed two points above 10% in the TRβ bioassay suggesting at least a partial agonistic activity towards the TRβ. In a general way, even though at-RA and 2-AAF were slightly active on the TRβ CALUX bioassay, we were not able to classify them with certainty due to the lack of available data at the time of the study. Overall, besides these six compounds (2-AAF, at-RA, T3, T4, TETRAC and TRIAC), all chemicals tested scored negative when assessed for TRβ agonistic activity. Although only few reports have related TRβ agonists, most of these negatively scored compounds were clearly reported antagonists and thus, were expected to be inactive in the agonist TRβ bioassay. As examples, amiodarone, BPA and TBBPA were all reported TRβ antagonists in literature, as well as the well-established antagonist reference compound 1-850 (Drvota et al. 1995; Moriyama et al. 2002; Schapira et al. 2003; Sun et al. 2009). No data were available regarding agonistic activity towards TRβ of the aflatoxin B1, T2-toxin, hydroquinone or valproic acid preventing us from comparing our results with existing data. Whereas these compounds require a closer examination to be part of the evaluation, we still classified them as inactive for the agonistic TRβ bioassay.

Overall, out of eighteen compounds tested, half (2-AAF, at-RA, aflatoxin B1, dieldrin, hydroquinone, methoxyacetic acid, T2-toxin, valproic acid and vinclozolin) were not used in predictivity and accuracy calculations due to the lack of reference data. For the compounds for which reference values were available, the agonist mode of the TRβ-CALUX bioassay showed a predictivity and accuracy of 100% (9/9). This study allowed the classification of yet unknown compounds, dieldrin, hydroquinone, methoxyacetic acid, T2-toxin, valproic acid and vinclozolin as non-responders and 2-AAF and at-RA as a weak TRβ agonist. It is not excluded that 2-AAF might act using an RXR-like dependent pathway, since it showed a similar dose-response curve as at-RA. Although we used a limit set of compounds, the results obtained from this intra-laboratory appraisal were promising and consistent with currently available data.

Outcomes acquired through the anti-TRβ CALUX evaluation showed a good correlation with existing data. In line with previously reported results, compound 1-850, BPA and TBBPA all scored positive in the antagonistic TRβ CALUX bioassay (Moriyama et al. 2002; Schapira et al. 2003; Sun et al. 2009). Alongside with the known antagonist deoxynivalenol, 4-nonylphenol and TBBPA showed a complete inhibition of the TRβ receptor activation resulting in a full dose response curve (Demaegdt et al. 2016). Surprisingly, at-RA was active on both agonist and antagonist modes of the TRβ CALUX bioassay. Amiodarone and zearalenone also showed TR antagonistic activity in the TRβ CALUX bioassay. Previous studies reported these

two compounds as antagonist of the TRβ which matched our results (Demaegdt et al. 2016; Drvota et al. 1995; Kiss et al. 2018). Interestingly, whereas mycotoxins like deoxynivalenol and zearalenone were already reported as TRβ antagonist, no prior data about were found T2-toxin at the time of writing (Demaegdt et al. 2016; Kiss et al. 2018). According to our results, T2-toxin is the most potent compound from the tested set (log IC_{50}=8.47). Along with deoxynivalenol and zearalenone, mycotoxins seem to present strong endocrine disrupting activities targeting TRβ. In this way, mycotoxins including T2-toxin will be of particular interest for further studies. Contrary, dibutylphthalate, endosulfan, pinoresinol and T3 were all found not to be antagonistic in the TRβ-CALUX bioassay. Whereas dibutylphthalate, endosulfan and pinoresinol were reported as antagonists in former studies no activity was measured in our bioassay (Kiss et al. 2018; Ogungbe et al. 2014; USEPA 2020). No data allowed us to confirm the antagonistic potential of these two compounds neither of at-RA, aflatoxin B1 nor T2-toxin. In this respect, these three compounds were excluded from predictivity calculations. Overall, the intra-laboratory evaluation demonstrated a good consistency with a global accuracy of 75% (9/12).

Used in combination with the TRβ CALUX, the TTR-binding assay reported consistent results based on comparison with existing data. In 1999, Meerts *et al.* classified TBBPA and TCBPA as T4 competitors in a radioactivity-based study, which entirely matched with our results (Meerts et al. 2000). In 2011, Cao *et al* assessed BPA using fluorescence probes and reported it as a weak competitor for TTR. In the present study, this compound also demonstrated capacity to compete with T4 at high concentration (1E-4M and higher) in the TTR-TRβ CALUX bioassay. As a whole, although we were not able to define an IC_{50} value, BPA matched our acceptance criteria of at least 20% diminution of TRβ activation and thus, was classified as a T4-TTR competitor (Cao et al. 2011). 4-nonyphenol, DES, PBP and PCP also showed T4-TTR binding competition activity with a calculated IC_{50} in the same range as data found in prior TTR-related studies (Ishihara et al. 2003; Meerts et al. 2000; Simon et al. 2013; van den Berg 1990). Perfluorinated chemicals PFOA and PFOS showed a high potency to bind TTR, confirming what has already been reported in prior study using a combination of TTR-binding and radiolabeled assays (Weiss et al. 2009). T2-toxin based measurements gave an IC_{50} of 1.00E-06M suggesting competitive properties towards T4, which has never been reported in literature at the time of the study. In contrast, alachlor is known not to compete for TTR-binding which was coherent with our results generated using the TTR-TRβ CALUX (Cheek et al. 1999). TTR seems to be an important target for compounds who interfere with the thyroid system, emphasizing the importance of developing test tool for assessing potential T4 competitors. Results obtained from our study demonstrated the reproducibility and stability of the TTR-TRβ CALUX while showing a good correlation with existing data.

The newly developed panel allows the assessement of yet unknown chemicals towards TRβ, as well as their possible interferences with T4/TTR binding. Using TRβ CALUX bioassay, a compound can be assessed for both agonistic and antagonistic activities. With the natural thyroid hormone T3 as a reference, an increase of at least 10% in TRβ activation allows to score the chemical as a TRβ agonist. Based on PC_{10} or EC_{50} values, it is possible to calculate the compound's relative potency expressed as a percentage of the reference's activity. A relative potency value above 1 suggests that the compound is more potent than the natural ligand while if below 1, a higher concentration is required to affect TRβ the same way as T3. This calculation provides an easy way to determine the potency of a new chemical, and to compare it with other compounds or existing data. In a similar way, antagonistic potential can be assessed in the TRβ CALUX bioassay. A decrease in TRβ activiation of more than 20% of the maximal effect of the reference compound deoxynivalenol suggests that the test compound presents antagonistic properties towads the receptor. Relative potency can be calculated based on either PC_{80} or IC_{50} values, allowing to compare the unknown chemical potency to others. A new chemical can be also assessed for T4 competition regarding TTR binding using the TTR-TRβ CALUX bioassay. Two points below 80% of the reference TBBPA's activity implies a competitive activity from the unknown compound. Its potency can be calculated and expressed as a TBBPA's potency percentage using PC_{80} or IC_{50} values. Overall the present panel is able to generate data regarding direct activity towards TRβ, while giving informations about their potential capacity to bind TTR thus, to pass through the blood brain barrier and the uterine-placental wall.

The demonstration study using water samples as an example, showed a positive case regarding potential thyroid hormones disrupting compounds activity in wastewater. No sign of TTR-binding potency was registered in HPLC water. An interesting follow-up of this case-study would be to collect waste water samples at different stages of treatment in a waste water treatment plant and analyze them in all variants of the panel in order to first determine the agonistic or antagonistic potential of these samples and secondly, evaluate the efficiency of various treatment procedures.

Overall, the *in vitro* TTR-TRβ CALUX assay appears to be a simple efficient method for assessing TTR binding disruption, giving a cheaper and safer alternative to other currently available bioassays mostly using radioactivity. This system was added to the (anti-)TRβ CALUX set so as to develop a mini test panel to assess the thyroid disruptive potential of various compounds at multiple levels. Also, the proposed battery can be performed alongside with the AR- and ER- CALUX bioassays in order to cover a variety of endpoints related to EATS (Estrogens Androgens Thyroid and Steroidogenesis) based-endocrine disruption.

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Possible Role of Per- and Polyfluoroalkylated Substances (PFAS) in the Thyroid-Related Endocrine Activity Observed in Breast Milk Samples from the Norwegian HUMIS Cohort

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Manuscript in preparation

Abstract

Per- and polyfluoroalkyl substances (PFAS) have been detected in body fluids (e.g. serum, cord blood, breast milk) and were hypothesised to interfere with the thyroid hormone (TH) system in plasma, by disrupting the transthyretin (TTR)-based TH-transport. In the present study, we investigated the effects of a set of PFAS compounds on the thyroid system by evaluating their capacity to compete with thyroxine (T4) for binding and transport on TTR as well as directly interacting with the thyroid receptor β (TRβ) in target cells. We also investigated whether human breast milk samples would contain TH-disrupting activity possibly associated with the presence of PFAS and related TH disrupting contaminants. Thirteen PFAS compounds were evaluated for their potential interactions with TRβ using the in vitro TRβ CALUX® reporter gene assay. This same set of compounds was subsequently analyzed on the TTR-TRβ CALUX assay to investigate the binding competition potency of each chemical compared to T4 to TTR. Subsequently, possible endocrine active compounds (EACs), including PFAS, were extracted from ten breast milk samples collected from mothers participating in the Norwegian HUMIS birth cohort. Extracts were analyzed in the TRβ- and TTR-TRβ CALUX assays. The concentration of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) in each sample was used to evaluate their possible contribution to the observed activity. None of the tested PFAS compounds demonstrated a direct agonistic activity towards the TRβ receptor in the TRβ CALUX reporter gene assay. However, seven PFAS compounds showed antagonistic activity in the anti-TRβ CALUX assay. All the PFAS compounds showed competitive binding properties with T4 on TTR using the TTR-TRβ-CALUX assay, albeit with different potencies. Antagonistic, but no agonistic activity, was observed in most breast milk extracts in the TRβ CALUX assay, with a potency equivalent to 0.41-3.6 μg deoxynivalenol eq./g of milk. Measurements demonstrated that all samples but one showed T4-TTR binding competitive potency, ranging from 0.41 to 9.1 μg PFOA eq./g of milk however, only a small percentage of the activity could be explained by PFAS. These results showed that PFAS are potent disruptors of the thyroid system by being able to compete with TH for TTR-transportation and by antagonizing TRβ activation. Moreover, our study indicates that breast milk from Norwegian mothers commonly have thyroid-disrupting activity, which can only partly be explained by the content of PFAS in milk.

Introduction

Poly- and perfluoroalkyl substances (PFAS) are industrial surfactants commonly used in a myriad of commercial products, e.g., textiles, food packaging, fire-fighting foam since the 1950s (Kissa 2001). PFAS are toxic at low concentration for both humans and animals, this together with the fact that they are practically non-biodegradable and extremely persistent in the environment, has lead to the implementation of manufacturing limitations under the Stockholm Convention on Persistent Organic Pollutants (Stockholm Convention 2008) in 2009 and by the US Environmental Protection Agency in 2013 (US EPA 2013). Despite these restrictions in manufacturing and use, humans and wildlife continue to be affected by PFAS accumulated in sediments, agricultural soil, ground- and surface water, contributing to the increase of body burdens and associated deleterious effects (Skutlarek et al. 2006; Zareitalabad et al. 2013).

The majority of PFAS including the most studied ones, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), share a polar hydrophobic structure allowing their interaction with various binding proteins e.g. albumin and globulins (Luebker et al. 2002). Over the past decades, toxicological studies, using rodents and primates, have shown the impact of PFAS on the thyroid hormone (TH) system, such as a significant decrease in circulating TH levels (Chang et al. 2008; Thibodeaux et al. 2003). Further investigations proved PFAS to be able to disrupt transport of the natural ligand 5',3',5',3-tetraiodo-[L]-thyronine (thyroxine, T_4) by TTR (Weiss et al. 2009). T_4 and its bioactive form 3',5,3-triiodo-[L]-thyronine (triiodothyronine, T_3) are essential in the activation of the thyroid hormone receptor alpha (TRα) and beta (TRβ) (Kim and Cheng 2013). Upon activation, these nuclear receptors modulate the transcription of thyroid-responsive genes essential for e.g., proper fetal development. Being the only thyroid transporter capable of mediating the delivery of TH to the fetus across the uterine-placental wall and the blood-brain barrier, TTR constitutes an essential actor of fetal maturation and a highly-sensitive target of endocrine active chemicals (EDCs) (Bergman et al. 2012; Landers et al. 2013).

Over the course of the last years, PFAS concentrations have been measured in various human fluids including cord blood, plasma and breast milk (White et al. 2011). Mogensen et al. (2015) and Papadopoulou et al. (2016) showed that serum-PFAS levels in children were co-incidentally increasing with the period of breastfeeding, indicating that breast milk is an important source of PFAS exposure in infants. More recently, evidence from an epidemiological study conducted in a Danish Birth Cohort showed associations between PFAS exposure and thyroid-stimulating hormone (TSH) levels in maternal blood samples (Inoue et

al. 2019). These findings indicate a transfer of the mother's load of PFAS to the offspring through body fluids, which may lead to a disruption of the thyroid system in the offspring.

Fluctuations in TH circulating concentrations is the major studied endpoint with respect to the thyroid system. To date there is relatively little data assessing direct interactions of PFAS with thyroid receptors. While TRα is predominant in the heart and brain tissues, TRβ is more widely distributed across the body and was proven to cause abnormal thyroid functions in patients with TRβ-resistance, indicating that this isoform could be a key element in PFAS-driven thyroid disruption (Ortiga-Carvalho et al. 2014). As a first step to investigate PFAS potential interaction with thyroid receptors, we examined the potential agonistic and antagonistic effects of a set of thirteen PFAS pure compounds against the nuclear receptor TRβ using the highly selective TRβ CALUX® reporter gene assay. Subsequently, we confirmed PFAS competitive properties towards T_4 using the combination of T_4-TTR-binding and TRβ CALUX® assays, developed and described earlier (Collet et al. 2019). In addition, a small pilot study was performed using ten breast milk samples originating from Norwegian mothers participating in the HUMIS birth-cohort study. Endocrine active compounds (EACs), including PFAS and endogenous hormones, were extracted from each breast milk sample. Extracts were analyzed to evaluate their potential disrupting activity towards TRβ and T_4 competitive properties for TTR binding, using the TTR-TRβ CALUX assays. Finally, the extent of daily exposure of a child to thyroid-disrupting chemicals through breastfeeding was estimated.

Material and methods

Chemicals

Thirteen PFAS were selected for evaluation of their potency towards TRβ and their binding competition activity for T_4 on TTR. Purchase information as well as abbreviations are detailed in Table 1. For all chemicals, 0.1M stock solutions were prepared in DMSO. Bisphenol A (BPA) (CAS no.: 80-05-7), deoxynivalenol (DON) (CAS no.: 4330-21-6), 17β-estradiol (estradiol) (CAS no.: 50-28-2), testosterone (CAS no.: 58-22-0), triiodothyronine (T_3) (CAS no.: 6893-02-3) and thyroxine (T_4) (CAS no.: 51-48-9) were purchased from Sigma-Aldrich.

Table 1. Information about the poly- and perfluoroalkyl substances selected for thyroid-related bioassays testing.

Compound		CAS number	Mol. weight (g/mole)	Manufacturer
PFBA	Perfluorobutyric acid	375-22-4	214.04	Campro Scientific
PFPeA	Perfluoropentanoic acid	2706-90-3	264.05	Campro Scientific
PFHxA	Perfluorohexanoic acid	307-24-4	314.05	Fluorochem
PFHpA	Perfluoroheptanoic acid	375-85-9	364.06	Sigma-Aldrich
PFOA	Perfluorooctanoic acid	335-67-1	414.07	Sigma-Aldrich
PFNA	Perfluorononanoic acid	375-95-1	464.08	Sigma-Aldrich
PFDA	Perfluorodecanoic acid	335-76-2	514.09	Sigma-Aldrich
PFBS	Perfluorobutanesulfonic acid	375-73-5	300.10	Sigma-Aldrich
PFHxS	Perfluorohexanesulfonic acid	355-46-4	400.11	Campro Scientific
PFHpS	Perfluoroheptanesulfonic acid	375-92-8	500.13	Dr. Ehrenstorfer
PFOS	Perfluorooctanesulfonic acid	1763-23-1	450.12	Dr. Ehrenstorfer
H4PFOS	1H,1H,2H,2H-Perfluorooctanesulfonic acid	27619-97-2	428.16	Dr. Ehrenstorfer
PFOSA	Perfluorooctanesulfonamide	754–91-6	499.14	Dr. Ehrenstorfer

Human milk samples

Breast milk samples were randomly selected from the mother-child birth-cohort study HUMIS (Human Milk Study), previously described by Eggesbø et al. (2009). HUMIS is a population-based birth-cohort study conducted between 2002 and 2009. Women involved in the study were asked to collect 25 mL of milk every morning for eight consecutive days between the first two weeks and two months after giving birth. Mothers were encouraged to avoid electrical pumping equipment and to report details regarding method and time of sampling. Milk samples were collected in 250 mL natural HDPE Packaging Bottles (Cat. no.: 967-21244, Thermo Scientific Nalgene®) made from plasticizer-free high-purity resins and posted by the

mothers. Bottles were stored at -20°C in a Biobank of the Norwegian Institute of Public Health upon arrival. The study was approved by the Norwegian Data Inspectorate (ref. 2002/1398) and Regional Ethics Committee for Medical Research (ref. S-02122).

PFOS and PFOA concentrations from HUMIS cohort samples were analyzed at the Research Centre for Toxic Compounds in the Environment, Masaryk University in the Czech Republic during a previous study (Forns et al. 2015). From the total biobank of breast milk samples, ten samples were included randomly.

Sample preparation

Endocrine active compounds (EACs) were extracted according to their polarity (apolar and polar) and combined to obtain a mixture closer to the original sample.

Polar compounds from breast milk samples were extracted according to a method described earlier (Collet et al. 2020). In short, a QuEChERS (Quick Easy Cheap Effective Rugged and Safe) solid phase extraction and cleaning method was slightly modified to allow the extraction of polar analytes from breast milk samples (Anastassiades et al. 2003). Samples were homogenized and 5 mL were transferred to a clean 50 mL tube Greiner and 15 mL of acetonitrile (ACN) (CAS no.: 75-05-8, BioSolve) were added. After quick homogenization one QuEChERS EN 15662 extraction packet (Cat. no.: 5982-5650, Agilent) was added. The tubes were shaken for 15 min using a circular shaker prior to a five-minute centrifugation at 4000 rpm, 4°C. The upper liquid layer was transferred to a clean tube and the solid lower phase mainly constituted of salts and water was re-dissolved by adding 15mL of ACN. After addition of an extra extraction packet, tubes were shaken and centrifuged one more time following the same procedure. Upper layers were combined and evaporated until ± 8 mL. The remaining volume was transferred to a 15 mL QuEChERS dispersive solid phase extraction (d-SPE) (Cat. no.: 5982-5158, Agilent) clean-up tube and vortex for 1 min. The d-SPE tubes were centrifuged for 5 min at 4000 rpm at 4°C and the upper layer was collected and evaporated until dryness using a gentle flow of nitrogen. All extracts were reconstituted in 30 µL of DMSO and stored at -20°C until analysis.

Procedure control consisting of 5 mL of spiked breast milk was included to assess the efficiency of the extraction. 100 µL of a mixture of internal standards of bisphenol A (CAS no.: 80-05-7, Sigma-Aldrich) (100 µg/mL), 17β-estradiol (E2) (CAS no.: 50-28-2, Sigma-Aldrich) (100 µg/mL) and testosterone (CAS no.: 58-22-0, Sigma-Aldrich) (100 µg/mL) was used, similar to the pilot study (Collet et al. 2020). Controls were analyzed by liquid chromatography using a Kinetex Biphenyl column (150x4.6mm 2.6µ particle size) (Cat. no.: 00F-4622-E0,

Phenomenex) following the procedure detailed earlier (Collet et al. 2020). Recovery values were evaluated by comparing the peak height in the control samples with the initial internal standard solution. BPA, E2 and testosterone were extracted with a recovery of 35±13, 50±6.8 and 59±5.9%, respectively.

Regarding apolar compounds, breast milk samples were first homogenized, and 5 mL was transferred to a clean 60 ml glass tube filled with 5 mL of 2-propanol (CAS: 67-63-0, BioSolve). After ten-minute shaking (200±20 strokes per minute), 14 mL of n-hexane (CAS: 110-54-3, BioSolve) was added and tubes were shaken for an extra hour. The upper layer was collected and transferred to a clean collecting tube. Another volume of n-hexane was added to the remaining phase and tubes were put back to shaking for 30 min. Upper layers were pooled and evaporated until dryness under a gentle flow of nitrogen. Samples were reconstituted in 1 mL of n-hexane and cleaned-up using 5 g of 2% deactivated silica column preliminary conditioned with 12 mL of n-hexane. Samples were eluted with 30 mL of a combination of n-hexane and dichloromethane (CAS no.: 75-09-2, BioSolve) to a ratio of 3:1. Solvents were evaporated until dryness and the final extract reconstituted in 30 µL of DMSO and stored at -20°C until analysis.

A set of controls consisting of 5 mL of breast milk primarily spiked with 50 µL of a solution of ^{13}C-labeled internal standard containing PCB153 and PCB180 (200 ng/ml) (MBP-D7, Wellington Laboratories) were also analyzed. Controls were processed as described earlier (Collet et al. 2020). In brief, controls were extracted following the same procedure as the samples with the exception that the final fraction was reconstituted in isooctane (CAS no.: 540-84-1, BioSolve). Recoveries were evaluated using a gas chromatograph/mass spectrometer (GC-MS) system using gas chromatograph GC-2010 Plus and gas chromatograph mass detector GCMS-TQ8050 (Shimadzu) controlled by the program GCMS Real Time Analysis (Shimadzu) and a CTC CombiPal autosampler controlled by the software Cycle Composer (Agilent Technologies). Parameters and settings used for the analysis were similar to those described by (Collet et al. 2020). Overall, recovery values were evaluated to be 96±13 and 115±21% for PCB153 and PCB180, respectively.

TRβ CALUX® reporter gene assay

TRβ CALUX reporter gene assay is based on human osteoblastic osteosarcoma U2-OS cells (American Type Culture Collection) stably transfected with a full-length human TRβ expression vector (pSG5-neo-hTRβ) and a luciferase reporter construct containing two copies of thyroid responsive elements (TRE) (Pgl3-2xTRE-Luc). The agonistic (TRβ CALUX) and antagonistic (anti-TRβ CALUX) versions of the bioassay were essentially performed as

described previously (Collet et al. 2019). In short, a suspension of TRβ cells (1x10^5 cells/mL) was prepared in assay medium consisting of DMEM/F12 without phenol red indicator (Cat no.: VX1041025, Fisher) supplemented with 10U/mL penicillin and 10 µg/mL streptomycin (P/S) non-essential amino acids (NEAA) (Cat no.: 11140-03, Gibco) and 5% charcoal-stripped fetal calf serum (DCC). Cells were seeded in 96-well plates and incubated for 20±4 hours at 37°C and 5% CO_2. On the following day, cells were exposed to serial dilutions of the test item in exposure medium consisting of assay medium without DCC. Pure chemicals were diluted following a 0.5 log unit increments method. To perform the antagonistic mode of the TRβ CALUX, exposure medium was supplemented with a fixed concentration of T_3 (EC_{50}; 1.7E^{-9}M in the well). Plates were placed back into the incubator for 22±2 hours. On the third day, medium was discarded and replaced by 30 µL/well of Triton-lysis buffer and plates were shaken for 10 mins. Luciferase signal in cellular lysates was measured using a luminometer InfinitePro coupled to a Connect Stacker (TECAN). A ten-point calibration line of the reference compound, T_3 for agonist mode and DON for antagonistic mode, was added to each plate (Collet et al. 2019).

TTR-TRβ CALUX assay

The TTR-TRβ CALUX assay was essentially performed as described earlier (Collet et al. 2020) with the exception that, for the purpose of the study, PFOA was used as the reference compound. Prior to analysis, dilution series from each PFAS stock solution (0.1M) were prepared (x1-3-10-30-100-300-1000-3000-10000-30000). Each concentration was incubated overnight at 4 °C in Tris buffer supplemented with a fixed concentration of T4 (0.05 µM in incubate) and TTR (0.06 µM in incubate) (CAS no.: 87090-18-4, Sigma Aldrich). Tris buffer consists of a mixture of Tris buffer (CAS no.: 77-86-1, Sigma Aldrich), NaCl (CAS no.: 7647-14-5, Sigma Aldrich) and ethylenediaminetetraacetic acid (CAS no.: 60-00-4, Sigma Aldrich) diluted in HPLC water (J.T Baker) and adjusted to pH 8.0. The next day, T_4- and PFAS-bound TTR complexes were separated from free T_4 molecules using self-made Bio-Gel P-6DG (Cat no.: 150-0739, BioRad) columns. Eluates resulting from this separation were taken-up in 500 µL of exposure medium (final volume 640 µL) and immediately analyzed on the TRβ CALUX reporter gene assay.

A dose-response curve of the reference compound PFOA was added to each 96-well plate. One negative control consisting of the highest concentration of the tested chemical without the addition of T4 in incubate, was added to each procedure. PFAS were tested in duplicate in two independent runs.

PFAS cytotoxicity in the TTR-TRβ CALUX were evaluated assuming that all PFAS molecules bonded to TTR and ended up in the incubate. For each compound, the highest concentration analyzed on the TTR-TRβ CALUX (0.1M), equivalent to $4.1E^{-4}$M in incubate, was also tested on the Cytotox CALUX bioassay. None of the tested PFAS concentration was sufficient to cause cytotoxicity in the TTR-TRβ CALUX bioassay.

Data analysis

Luciferase measurements were corrected for background signal using pure DMSO as a blank. Activity derived from each test item was expressed as relative induction to the maximum signal response (100% for agonist mode, 0% for antagonist) set by the reference compound. Results from each pure chemical or epidemiological sample were interpolated in the associated calibration curve using the statistical software package GraphPad Prism V5.03 (non-linear regression, variable slope, 4 parameters, robust fit). Using the same program, EC_{50} (i.e. 50% of the maximal effective concentration) (agonist) or IC_{50} (50% of the maximal inhibitor concentration) (antagonist) were calculated. For measurements on the TRβ CALUX assay, a compound was defined as positive if two consecutive points reached PC_{10} (i.e. increase of at least 10% in receptor activation) or PC_{80} (i.e. decrease of at least 20% in receptor activation) for agonist and antagonist mode, respectively. Data derived from TTR-TRβ CALUX analysis were handled the same way as the antagonistic TRβ CALUX bioassay, using PC_{80} as a threshold.

Results

PFAS agonistic and antagonistic activity towards TRβ

The agonistic and antagonistic potential of selected PFAS were individually investigated using the (anti-)TRβ CALUX bioassays (Table 2.). Measurements showed that none of the compounds led to TRβ activation. In contrast, more than half of the tested PFAS (PFHpA, PFHxA, PFHpA, PFHxS, PFHpS, PFOS, H4PFOS, PFOSA) demonstrated antagonistic properties towards TRβ. However, only three compounds, PFNA, PFDA and PFOSA, showed a full-dose response curve allowing the calculation of a log IC_{50} value. In conclusion, none of the PFAS from the test set was found to be TRβ agonist however, most of them were able to inhibit TRβ.

Table 2. Effect of PFAS congeners in the (anti-)TRβ CALUX bioassays, expressed as log EC_{50}/PC_{10} and IC_{50}/PC_{80} values.

	TRβ CALUX		**anti-TRβ CALUX**	
Reference compound	**LOG [EC_{50}]**	**LOG [PC_{10}]]**	**LOG [IC_{50}]**	**LOG [PC_{80}]]**
T_3	-9.00	-9.77		
DON			-6.16	-6.49
Test compound				
PFBA	NA	NA	NA	NA
PFPeA	NA	NA	NA	NA
PFHxA	NA	NA	NA	NA
PFHpA	NA	NA	NA	-5.12
PFOA	NA	NA	NA	NA
PFNA	NA	NA	-4.93	-5.17
PFDA	NA	NA	-5.43	-5.61
PFBS	NA	NA	NA	NA
PFHxS	NA	NA	NA	-4.8
PFHpS	NA	NA	NA	NA
PFOS	NA	NA	NA	-5.15
H4PFOS	NA	NA	NA	-4.38
PFOSA	NA	NA	-5.28	-5.40

Note: NA: no activity, value could not be calculated.

PFAS competitive potency for T4-TTR binding

Thirteen PFAS were individually tested in the TTR-TRβ CALUX assay (Figure 1 a.b). All tested PFAS compounds showed capacities to interfere with the thyroid system. Using PFOA as a reference and IC_{50} as a basis, the relative potency (REP) of each PFAS was evaluated (Table 3.). These calculations ranked PFHxS, PFHpS and PFOS as the most potent compounds among the tested PFAS with a REP value equal to 2.7, 1.7 and 2.2, respectively. On the contrary, PFBA, H4PFOS, and carboxylic-based PFAS showed a REP value ≤0.94, i.e., lower than the PFOA binding potency.

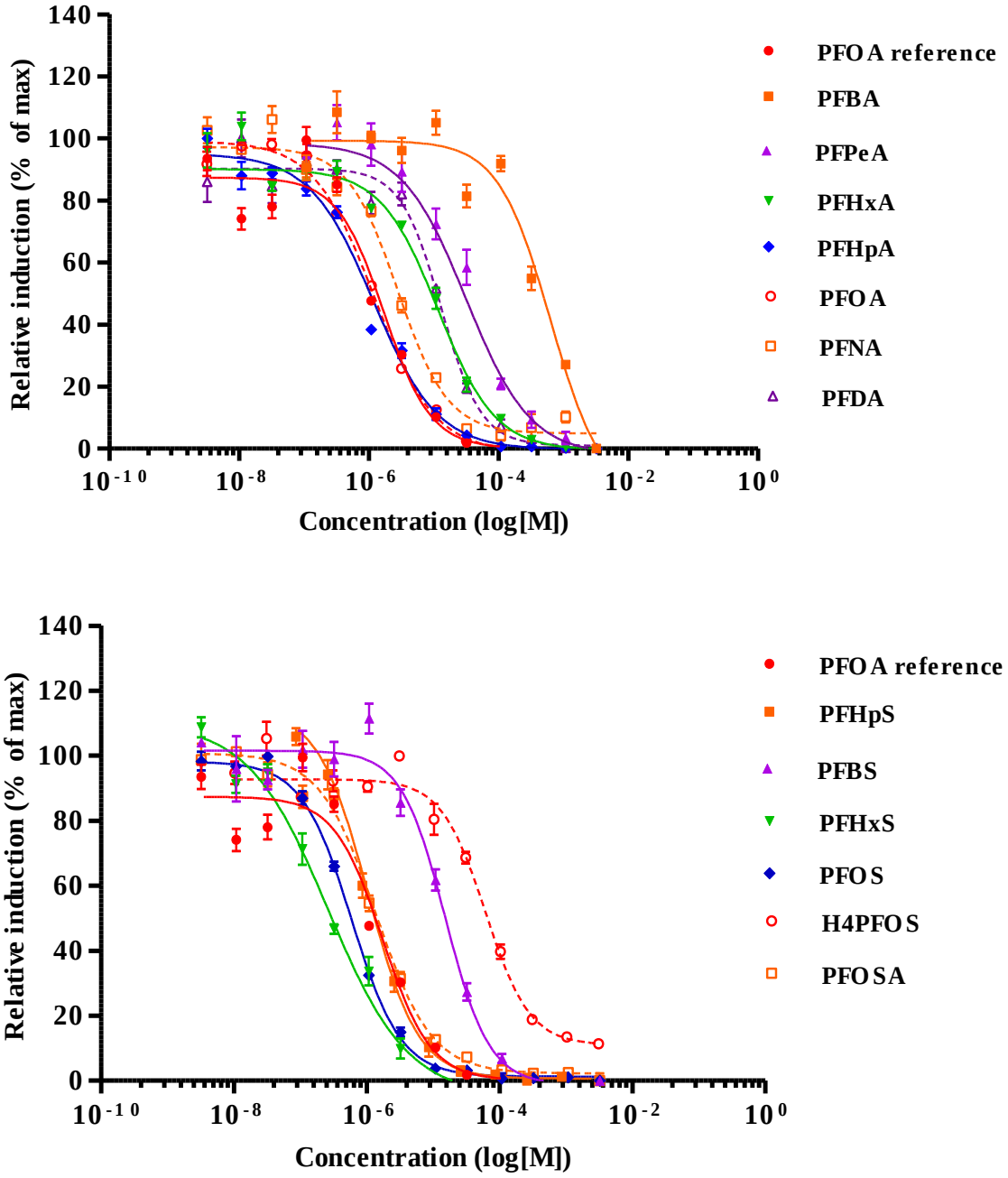

Figure 1.a.b. Dose-response curves for thirteen PFAS tested on the TTR-TRβ CALUX assay.

Note: PFAS were tested in duplicate in two independent runs. Each point represents the average of these measurements.

PFOA: Perfluorooctanoic acid; PFBA: Perfluorobutyric acid; PFPeA: Perfluoropentanoic acid, PFHxA: Perfluorohexanoic acid; PFHxA: Perfluoroheptanoic acid; PFNA: Perfluorononanoic acid PFDA: Perfluorodecanoic acid. PFBS: Perfluorobutanesulfonic acid; PFHxS: Perfluorohexanesulfonic acid; PFHpS: Perfluoroheptanesulfonic acid; PFOS: Perfluorooctanesulfonic acid; H4PFOS: 1H,1H,2H,2H-Perfluorooctanesulfonic acid; PFOSA: Perfluorooctanesulfonamide

Table 3. log IC_{50}, log PC_{80} and IC_{80}-based relative potency values for thirteen PFAS tested in TTR-TRβ CALUX assay.

Compound	LOG [IC_{50}]	LOG [PC_{80}]]	Relative Potency (REP)
PFOA (reference)	-5.81	-6.47	1
PFBA	-2.94	-4.01	0.00091
PFPeA	-4.45	-5.11	0.035
PFHxA	-4.98	-5.77	0.12
PFHpA	-5.92	-6.69	0.94
PFOA	-5.92	-6.55	1.0
PFNA	-5.50	-6.01	0.35
PFDA	-4.86	-5.58	0.088
PFBS	-4.79	-5.25	0.075
PFHxS	-6.37	-6.92	2.7
PFHpS	-6.15	-6.69	1.7
PFOS	-6.25	-6.78	2.3
H4PFOS	-4.28	-4.76	0.024
PFOSA	-5.86	-6.44	0.90

Note: IC_{50}: concentration at 50% TRβ inhibition
PC_{80}: concentration at a decrease of 20% of TRβ activation
REP based on IC_{50} values

Antagonistic effects towards TRβ on breast milk samples

A total of ten breast milk sample extracts were individually screened in the agonistic and antagonistic mode of the TRβ CALUX reporter gene assay (Table 4). Samples were analyzed in three fractions: apolar endocrine active compounds (EACs), polar EACs and reconstituted mixture consisting of the combination of apolar and polar EACs. All samples regardless of the fraction were below the limit of quantification (LOQ) (≤0.28) of the TRβ CALUX bioassay in agonistic mode. In contrast, more than half of the polar fractions scored positive on the anti-TRβ CALUX, with an activity ranging from 0.86 to 2.02 μg DON eq./g of milk. The apolar fractions did not show activity above the LOQ. All reconstituted samples (i.e. mixtures) but two showed antagonistic activity towards TRβ, equal to 0.91-3.6 μg DON eq./g of milk.

Table 4. (Anti-)TRβ CALUX activity assessment in mixture, apolar and polar fractions from human milk samples.

Sample number	TRβ CALUX (µg T3 eq./g of milk)			Anti-TRβ CALUX (µg DON eq./g of milk)		
	Mixture	**Apolar**	**Polar**	**Mixture**	**Apolar**	**Polar**
1	LOQ (<0.17)	LOQ (<0.24)	LOQ (<0.17)	1.2	LOQ (<0.28)	LOQ (<0.28)
2	LOQ (<0.24)	LOQ (<0.25)	LOQ (<0.23)	3.6	LOQ (<0.28)	0.87
3	LOQ (<0.23)	LOQ (<0.25)	LOQ (<0.23)	1.2	LOQ (<0.28)	0.86
4	LOQ (<0.23)	LOQ (<0.25)	LOQ (<0.23)	1.6	LOQ (<0.28)	1.2
5	LOQ (<0.23)	LOQ (<0.25)	LOQ (<0.23)	LOQ (<0.55)	LOQ (<0.54)	1.5
6	LOQ (<0.24)	LOQ (<0.26)	LOQ (<0.23)	1.9	LOQ (<0.24)	2.0
7	LOQ (<0.21)	LOQ (<0.26)	LOQ (<0.23)	1.4	LOQ (<0.24)	1.3
8	LOQ (<0.21)	LOQ (<0.26)	LOQ (<0.23)	0.91	LOQ (<0.28)	LOQ (<0.48)
9	LOQ (<0.21)	LOQ (<0.26)	LOQ (<0.23)	1.1	LOQ (<0.24)	LOQ (<0.48)
10	LOQ (<0.21)	LOQ (<0.26)	LOQ (<0.26)	LOQ (<0.55)	LOQ (<0.25)	LOQ (<0.47)
Average	LOQ	LOQ	LOQ	1.4	LOQ	0.94

Note: LOQ: Below the limit of quantification of the bioassay.

T_4-TTR binding competing activity in human milk samples

Extracts derived from ten breast milk samples were also individually analysed for interferences with TTR transportation using the TTR-TRβ CALUX bioassay (Table 5.). Most apolar fractions did not reach LOQ value (LOQ=0.03) with the exception of two samples demonstrating a very low signal (≤0.07 µg PFOA eq./g of milk). On the contrary, every polar fraction scored positive in the TTR-TRβ CALUX assay with an activity ranging from 1.3 to 11.5 µg PFOA eq./g of milk. Interestingly, mixtures showed different behaviours depending on the assessed sample. Indeed, four samples exhibited significantly higher activity in the mixture than in the apolar and polar fractions alone. In contrast, six mixtures demonstrated diminished effects compared to their corresponding single fractions. On average, thyroid disrupting properties measured in mixtures were slightly lower in comparison with polar measurements (3.4 µg PFOA eq./g of milk versus 4.6 µg PFOA eq./g of milk). Overall, all reconstituted human milk samples but one showed potential disrupting properties targeting the thyroid hormone transport system.

Subsequently, we estimated the contribution of PFOA and PFOS to the measured thyroid-interfering activity (Table 6). Sample n°5 is the only one with a higher REP ($7.02E^{-10}$ mol PFOA eq./L of milk) than overall activity (LOQ). In general, less than 1% of thyroid

disrupting activity in the measured TTR-TRβ CALUX assay could be explained by the presence of these two PFAS chemicals.

Table 5. Thyroid-disrupting activity in the TTR-TRβ CALUX bioassay from extracts of breast milk samples.

Sample number	TTR-TRβ CALUX (µg PFOA eq./g of milk)		
	Mixture	**Apolar**	**Polar**
1	0.67	LOQ (<0.03)	1.3
2	1.5	LOQ (<0.03)	3.7
3	3.7	0.04	3.2
4	3.3	LOQ (<0.03)	3.0
5	LOQ (<0.03)	0.07	3.2
6	2.9	LOQ (<0.03)	8.1
7	9.1	LOQ (<0.03)	4.7
8	6.7	LOQ (<0.03)	3.5
9	0.41	LOQ (<0.03)	2.1
10	2.1	LOQ (<0.03)	11.5
Average	3.0	0.035	4.4

Table 6. Estimation of thyroid-disrupting activity in the TTR-TRβ CALUX bioassay by extracts from breast milk samples based on earlier reported PFOA and PFOS concentration in those samples.

Sample number	Concentration (ng/L of milk)		Total activity* (mol PFOA/L of milk)	REP** (mol PFOA eq./L of milk)
	PFOS	**PFOA**		
1	46	16	1.6E-06	2.2E-10
2	150	30	3.0E-06	6.8E-10
3	53	23	8.8E-06	2.7E-10
4	180	90	8.0E-06	9.4E-10
5	120	90	LOQ (<0.55)	7.0E-10
6	130	42	6.9E-06	6.3E-10
7	69	16	2.2E-05	3.2E-10
8	150	60	1.6E-05	7.5E-10
9	49	-	9.9E-07	NA
10	150	70	5.1E-06	7.8E-10

Note: *based on disrupting activity of reconstituted mixtures
**Relative Potency based on PFOS/PFOA concentration in the sample
NA: not available, value could not be calculated

Estimation of daily intake of anti-thyroid chemicals through breastfeeding during the first year of life

The World Health Organization (WHO) strongly recommends exclusive breastfeeding for every child during their first year of life. According to reported guidelines, an infant should ingest approximatively 150 mL of milk per kg of body weight (bw) per day (WHO 2011). Previous findings showed that breast milk originating from Norwegian mothers for the HUMIS cohort could be contaminated with anti-TRβ toxicants as well EDCs with T4-TTR competing properties for TTR binding, equivalent to 1.4 µg DON eq./g of milk and 3.02 µg PFOA eq./g of milk, respectively. We further estimated a child's average exposure to thyroid-disrupting EDCs during the first year of life (Table 7, 8).

Table 7. Estimation of a child's daily intake of anti-thyroid EDCs through breastfeeding at one month, six months and twelve months.

Average activity Anti-TRβ CALUX (µg DON eq./mL)	Age (months)	Daily intake of milk (mL)*	Estimated intake per day (µg DON eq./day)	Body weight** (kg)	Nursing child dose per day (µg DON eq./kg bw/day)
1.4	1	525	735	3.5	210
1.4	6	1125	1575	7.5	210
1.4	12	1380	1932	9.2	210

Note: *Assuming that the child is exclusively breastfed.
** Average body weight at 1, 6 or 12 months.
DON: deoxynivalenol; T_3: triiodothyronine

Table 8. Estimation of a child's daily intake of TTR disrupting EDCs through breastfeeding at one month, six months and twelve months.

Average activity on TTR-TRβ CALUX (µg PFOA eq./mL)	Age (months)	Daily intake of milk (mL)*	Estimated intake per day (µg PFOA eq./day)	Body weight** (kg)	Nursing child dose per day (µg PFOA eq./kg bw/day)
3.02	1	525	1585	3.5	453
3.02	6	1125	3398	7.5	453
3.02	12	1380	4168	9.2	453

Note: *Assuming that the child is exclusively breastfed.
** Average body weight at 1, 6 or 12 months.
PFOA: perfluorooctanoic acid; T_4: thyroxine

Discussion

The present manuscript demonstrates the ability of PFAS to interfere with two biomolecular levels of the thyroid system, i.e., plasma transport and thyroid receptor inhibition. Seven PFAS (PFHpA, PFNA, PFDA, PFHxS, PFOS, H4PFOS and PFOSA) showed antagonistic activity towards TRβ, indicating that this nuclear receptor might be a potential target of PFAS-driven endocrine disruption. Furthermore, using the TTR-TRβ CALUX bioassay, we confirmed that individual PFAS have competitive properties towards T4 for binding to TTR, an important plasma carrier of thyroid hormones to e.g. the fetus. Further analysis showed that human milk sample extracts caused TRβ inhibition and T4-TTR binding competitive properties. However only 1 % of the observed TH disrupting activity by extracts from breast milk samples could be explained by the measured levels of PFOA and PFOS, thus the culprit(s) causing thyroid disruption is largely unknown.

None of the thirteen investigated PFAS were active as agonists in the TRβ CALUX reporter gene assay. A previous extensive screening performed amongst 8305 features demonstrated that only chemicals with high homology to thyroid natural ligands could potentially act as TRβ agonists (Paul-Friedman et al. 2019). On the contrary, seven showed antagonistic activity towards the same receptor. Results suggest that PFAS containing a carboxylic acid functional group and with a carbon chain length of nine or higher (PFNA, PFDA) are potent TRβ antagonists. Similarly, analysis of sulfonate based PFAS presenting eight carbons, PFOS (log PC_{80} -5.15) and H4PFOS (log PC_{80} -4.38), were also reported as antagonists although to a lower extend. Only in the case of PFOSA the dose-response curves reached an IC50 value (log IC50 -5.28) suggesting that the attached amine functional group could influence chemical potency. Overall, our data clearly reveal the capacity of some PFAS to act as TRβ antagonists, although their potency may vary depending on their structural properties.

Using PFOA as a reference compound, the TTR-TRβ CALUX analysis revealed that PFHpS, PFHxS and PFOS have a higher TTR binding potency (REP≥1.7) than carboxylic and other sulfonic compounds (REP≤1). These results are similar to the ranking reported by Weiss et al. (2009) placing PFHxS as the most potent of the test set, followed by PFOS. Overall, findings show that the TTR-disrupting potency is associated with PFAS with a carbon chain reaching a maximum length at eight carbons, PFOA (REP=1) and PFOS (REP=2.3). However, H4PFOS, a sulfonate PFAS with the same carbon chain length (n=8) but different degree of fluorination showed lower competitive binding properties (REP=0.024) in comparison with PFOS (REP=2.3). These findings confirmed the importance of the carbon chain length, degree

of fluorination and the attached functional group in TTR-binding competition potency, as reported earlier by Weiss et al. (2009).

While none of the breast milk extracts presented agonistic properties, antagonistic activity towards TRβ was detected in most breast milk samples (0.91-3.6 µg DON eq./g of milk). Based on these results, we estimated DON daily intake of a nursing child through breastfeeding to 210 µg DON eq./kg bw/day. All reconstituted mixtures of extracts from breast milk samples, except one, showed T4 competitive activity for TTR-binding ranging from 0.41 to 9.1 µg PFOA eq./g of milk. These results suggest that human milk from HUMIS participants contain compounds able to disrupt TRβ and TTR plasma T4-carrier by mimicking natural thyroid ligands. However, considering the myriad of chemicals present in body fluids, it is challenging to assess the importance of PFAS in these findings. Moreover, only two of the PFAS compounds, e.g., PFOA and PFOS concentrations were measured in the breast milk samples. Nevertheless, these findings lead to an estimation a nursing child's daily intake of 453 µg PFOA eq./kg bw/day. This value largely exceeds the tolerable PFAS daily intake of 0.63 ng/kg bw/day established in 2020 by the European Food Safety Authority Panel on Contaminants in the Food Chain (EFSA CONTAM Panel) (PFAS tolerable week intake: 4.4 ng/kg bw/week) (Schrenk et al. 2020). The fact that PFOA and PFOS could only justify a small fraction (1%) of the observed thyroid disrupting activity in breast milk, can partially be explained by the lack of information on the presence of the other PFAS compounds, which constitute a complex mixture containing more than 6000 congeners. Moreover, several other classes of contaminants are capable of interfering with the thyroid transport system, such as brominated flame retardants, dibenzo-p-dioxins (PCDDs), and organochlorine pesticides (aldrin, chlordane, α-endosulfan, methoxychlor, etcetera) which have been identified in milk from Norwegians mothers (Čechová et al. 2017; Iszatt et al. 2016; Maroni et al. 2000; Vollset et al. 2019). Overall, these results suggest an important contamination of Norwegian women's breast milk by anthropogenic toxicants, highlighting a possible threat for the nursing infant during the sensitive period of his first months of life. In future more work should be devoted to try to discern which culprit chemicals in breast milk are causing the thyroid disrupting activity.

Conclusion

In this manuscript we demonstrated that PFAS are potent disruptors of the thyroid system by being able to compete with T4 for TTR-transportation but also to antagonize TH for TRβ activation. This double hit could result in a significant drop of TH circulating levels and a reduced TRβ activity in target tissues. Overall, extracts of breast milk from the participating Norwegian mothers contain a clearly measurable potency to interfere with T4 binding to TTR,

as well as antagonizing TRβ suggesting that environmental EDCs, such as PFAS may be involved in this thyroid disrupting activity, which is of high concern due to the vulnerability of the newborn baby. Further studies are needed to identify the culprit chemicals causing the thyroid disruption, as well as human epidemiological studies focusing on possible thyroid-related adverse health outcomes in breast-fed infants.

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Summary, Conclusions and Future Outlook

This thesis investigates the presence and potential effects of endocrine disrupting chemicals (EDCs) in human milk using samples derived from the Norwegian HUMIS cohort. This study is based on a two-step approach involving firstly the extraction of hormonally active compounds according to their polarity. Secondly, apolar, polar and the combination of both extracts (mixture) were analyzed using *in vitro* human-based reporter gene CALUX bioassays covering key endpoints of the endocrine system: estrogens, androgens, and thyroid hormones (THs).

Chapter summary

Chapter one provides background on contaminants with endocrine disrupting properties while introducing the steroid- and thyroid signaling pathways. This chapter also details previous findings of the Norwegian HUMIS birth-cohort and the main objectives of the present thesis. In addition, due to their importance in the studies presented in this thesis, effect-based bioassays as well as effect-directed fractionation are introduced.

Chapter two constitutes a pilot study performed on ten human milk samples using the (anti-)ERβ and (anti-)AR CALUX bioassays. While nearly no estrogenic activity was detected, this work shows that all samples except one contained quantifiable amounts of anti-androgenic activity of unknown origin. Interestingly, measurements demonstrate higher activity in polar fractions in comparison to reconstituted apolar-polar extracts while none of the tested apolar fractions was active in the anti-AR CALUX bioassay. Further investigation involving effect-directed fractionation and exclusion methods showed that the observed anti-androgenic activity could not be explained by the presence natural hormones (androstenedione, DHEA, E1, E2, pregnenolone, progesterone, testosterone) and/or metabolites thereof. Therefore, these results suggest that the observed anti-AR activity in human milk samples is more likely to be caused by as yet unidentified, polar anthropogenic contaminants.

There is growing *in vitro* and *in vivo* evidence suggesting that early exposure to anti-androgens may result in reproductive impairments such as cryptorchidism (Borch, Metzdorff, Vinggaard, Brokken, & Dalgaard, 2006; Fisher, 2004; Welsh et al., 2008). Therefore, in **Chapter three**, 199 human milk samples originating from the HUMIS cohort, including a subset 94 mothers who gave birth to boys with undescended testis and a subset of 105 mothers 'controls' who gave birth to boys with no cryptorchidism, were selected to perform a case-study.

In this chapter a potential association between anti-androgenic activity, measured by the anti-AR CALUX in mother milk and prevalence of cryptorchidism in the offspring was evaluated. Although statistical analysis showed no significant difference between the human milk-based anti-AR activity measured in control and cryptorchid samples, a possible association between the presence of apolar compounds in human milk and undescended testis prevalence could not be ruled out. Overall, anti-androgenic activity was measured in the majority of the milk samples. Analysis of fractions with different polarity, i.e. apolar, polar and combined extracts, confirmed previous findings in chapter two, highlighting that polar compounds contributed most to the anti-androgenic activity in breast milk. While the origins of such compounds are still unclear, this study demonstrates the importance of assessing mixtures rather than investigating individual chemicals or subclasses.

Although sex steroids have been found to be particularly sensitive targets of endocrine disruption in early life, the past years raised an additional concerned regarding EDCs targeting the thyroid system, causing amongst other alterations of circulating thyroid hormone levels and interferences with the serum transporters of THs, in particular transthyretin (TTR). In **Chapter four**, the development and validation of a novel thyroid CALUX testing panel was evaluated, specifically designed to assess EDCs capabilities to interact with the thyroid receptor β (TRβ) (TRβ CALUX assay) and/or TTR (TTR-TRβ CALUX assay). The TRβ CALUX assay consists of an endogenously expressing TRβ expressing U2OS osteosarcoma cell line, which was stably transfected with a luciferase reporter gene, linked to thyroid responsive elements. This TRβ CALUX assay is capable of assessment of thyroid hormone receptor-based agonistic activity by quantification of the luciferase activity produced. The TRβ CALUX assay can also be used to measure antagonistic activity, through cell pre-stimulation with natural ligand T3 (anti-TRβ CALUX assay), or to evaluate T4 binding competition activities for TTR, when used in combination with the TTR binding assay. Each bioassay (TRβ and TTR-TRβ) was validated using a range of known positive and negative thyroid-disrupting compounds and showed a very good predictability suggesting that the newly developed CALUX panel can be a powerful tool in predicting the effects of EDCs on endogenous thyroid hormonal actions.

In **Chapter five**, the newly developed thyroid-specific CALUX assay panel, was used to investigate a set of per- and polyfluoroalkyl substances (PFAS), hypothesised to be thyroid disruptors. None of the tested PFAS showed agonistic activity towards TRβ in the TRβ CALUX reporter gene assay however, seven PFAS presented antagonistic activity on TRβ. TTR-TRβ-CALUX analysis showed that all PFAS have T4 competitive binding properties for TTR binding, albeit with different potencies. In this chapter, we also investigated whether human breast milk samples from the Norwegian HUMIS cohort contained TH-disrupting activity possibly

associated with the presence of contaminants, including PFAS. While no TRβ agonistic activity was detected, most breast milk extracts showed antagonistic activity towards TRβ and all of them but one presented T4-TTR binding competitive potency, equal to 0.41 to 9.1 µg PFOA eq./g of milk. Considering their abilities to compete with TH for TTR-transportation and to antagonize TRβ activation, results presented in chapter five suggest that PFAS can be potent thyroid disruptors. Although the sample size was quite small (ten samples), the pilot study performed on HUMIS samples showed the presence of thyroid-disrupting activity in extract from breast milk. Further calculations based on PFOA (perfluorooctanoic acid) and PFOS (perfluorooctane sulfonate) sample concentrations demonstrated that only a small percentage of the TRβ antagonistic activity could be explained by PFAS contamination, suggesting the additional presence of unknown thyroid-disrupting chemicals in human milk.

Conclusions and Future Outlook

Antiandrogenic activity of anthropogenic origin detected in Norwegian human milk samples

This research demonstrates for the first time the ubiquitous presence of anti-androgenic activity in human milk samples originating from the Norwegian HUMIS cohort. The studies conducted in Chapter two (pilot-study) and in Chapter three (case-control study), covering more than 200 participants, showed unexpected antagonistic activity towards the AR highlighting human milk contamination with as yet unidentified anthropogenic EDCs of high polarity. These findings suggest that breastfeeding may pose a potential route of early human exposure to mixtures of anti-androgenic contaminants.

In 1995, Sonawane (1995) established an extensive list of human milk contaminants identifying pesticides, polychlorinated biphenyls (PCBs) and dioxins as the most prominent compounds. Over the past decades, these toxicants were proven to interfere with the androgen signaling pathway via AR binding (Hotchkiss et al., 2004; Kelce, Gray, & Wilson, 1998; Van der Burg et al., 2010). Considering their physico-chemical properties, dioxins, dioxin-like PCBs and PCBs are unlikely to be responsible for the anti-androgenic activity measured in the polar fractions of milk samples (Sohoni & Sumpter, 1998; Sultan et al., 2001). On the contrary, high polarity pesticides e.g. aldrin, dieldrin, endrin, fenarimol, linuron and methoxychlor, as well as xenoestrogens may have contributed to the anti-AR activity found in the Norwegian human milk samples. More recently, some ester-based flame retardants were reported to also antagonize AR activation (Rosenmai et al., 2021). Although there are little to no toxicological data regarding their potential anti-androgenic activity, we cannot exclude that contaminants such as brominated flame retardants (BFRs) and phthalates esters, previously identified in

human milk, could also be potential actors in the measured anti-AR activity (Main et al., 2006; Norén & Meironyté, 2000; Thomsen et al., 2010). In the present thesis, we showed the presence of anti-androgenic activity in human milk extracts and were able to exclude a potential contribution of major sex hormones and their metabolites to the observed activity. Further calculations based on anti-AR CALUX findings estimated the daily exposure of a nursing child (1-12 months old) to 78 µg flutamide eq./kg of body weight/day. This level was found to largely exceed the NOAEL (no adverse effects level) of 0.025mg/kg/day reported in 2017 for humans (Zacharia, 2017). Although the estimated anti-androgenic activity is surpassing acceptable daily doses, it is of main importance to supplement this research with targeted and untargeted analysis in the near future to precisely characterize the chemical nature of the observed anti-AR activity in human milk in order to identify their origin and better advice regulatory authorities on necessary further chemical restrictions.

Investigation of a possible association between anti-androgenic activity in breast milk and prevalence of cryptorchidism based on a subset of 199 samples participants of the Norwegian HUMIS cohort

The case-control study presented in this thesis evaluates a possible association between anti-androgenic activity in human milk extracts and prevalence of cryptorchidism in children. Although no significant differences between case and control groups were observed, further statistical analysis revealed a possible, but yet non-significant, association between anti-AR activity from apolar compounds and undescended testis in the offspring. There are various limitations which may have hampered our study and reduced its statistical power. Firstly, the limited sample size (199 samples) might not have been enough to detect a possible relationship, if any, between anti-androgenic activity in milk and the prevalence of cryptorchidism in human offspring. Moreover, while breast milk is usually defined as a suitable matrix for monitoring late fetal exposure e.g., based on a proven correlation between breast milk and umbilical cord concentrations of persistent chemicals, important stages of the sexual development occurring during earlier fetal developmental windows (8 – 10 weeks) might have been overlooked (Kanja, Skaare, Ojwang, & Maitai, 1992; Verner et al., 2013; Waliszewski, Aguirre, Infanzon, Silva, & Siliceo, 2001). Lastly, a child's undescended, or late descendent testicle condition was based on self-observation and self-reported data by the mother, which can be inclined to bias. Therefore, it may require a larger case-control study, involving self-reported as well as medical professional-based reporting of cryptorchidism to more definitely be able to draw conclusions on a possible relationship between anti-androgenic activity in milk and the risk of cryptorchidism in human infants.

Several PFAS are potent T4-TTR binding competitors and TRβ antagonists

This thesis demonstrated PFAS ability to interfere with the thyroid system at several biomolecular levels, i.e. TTR-based plasma transportation and TRβ activation. Similar to previous findings reported by Weiss et al. (2009), TTR-TRβ CALUX analyses performed in chapter five revealed that PFHxS and PFOS have a higher T4 binding competition potency for TTR than their carboxylic and other sulfonic perfluorinated analogues. We also demonstrated that PFHpS was also a potent T4 competitor for TTR binding. In addition, an antagonistic activity on the TRβ was observed for several PFAS (PFHpA, PFNA, PFDA, PFHxS, PFOS, H4PFOS and PFOSA) to inhibit TRβ activation. To our knowledge, this is the first study reporting on TRβ antagonism by PFAS congeners. Results detailed in chapter five suggest a potential correlation between carbon chain length and functional group of the PFAS congeners and antagonistic potency towards TRβ. We demonstrated that perfluorinated carboxylic acids presenting a carbon chain length of nine or higher i.e. PFNA, PFDA, are potent TRβ inhibitors. To a lesser extend, perfluorinated sulfonic acids of eight i.e. PFOS and H4PFOS were also reported to antagonize TRβ activation. The high TRβ antagonistic potency of PFOSA, superior to other PFAS of the set, suggests a possible influence of the attached amine functional group in chemical thyroid-disrupting potency. Overall, we demonstrated that PFAS can be potent endocrine disruptors able to interfere at different biomolecular levels of the thyroid system. Besides their known T4-competitive binding interferences on TTR, i.e., the thyroid hormone transport protein facilitating T4 delivery to the fetus, PFAS were proven to influence TRβ activation implying a possible double hit for the growing fetus. Further investigation is necessary to better understand mechanisms underlying PFAS-based thyroid disruption. Moreover, complementary effect-based analysis and toxicological studies are required in order to establish or adjust existing exposure threshold limit values.

Thyroid-disrupting activities measured in extracts of human milk from the Norwegian HUMIS cohort, seem mostly independent from PFAS action

The second pilot case-study performed in this thesis highlighted the presence of thyroid-disrupting activities in milk samples from the HUMIS cohort. Out of ten participants, eight human milk TRβ CALUX measurements demonstrated antagonistic activity towards TRβ. Similarly, nine reconstituted mixtures showed competitive properties targeting the natural hormone T4 for TTR binding. The present work suggests that environmental contaminants, such as PFAS, are present in human milk and can be a possible source of exposure for a nursing child. Although chemical analyses performed and reported in earlier studies showed the presence of PFAS in most HUMIS milk samples (PFOS and PFOA), our work revealed that observed PFAS levels could only explain a trivial fraction of thyroid

disrupting activities measured in our study. While this result could be partially explained by the lack of information on the presence of other PFAS compounds in the milk, it is more likely that many other classes of contaminants e.g. organochlorine pesticides and BFRs, previously identified in HUMIS milk samples, may also have contributed to the measured anti-thyroid activity (Eggesbø et al., 2011; Forns et al., 2015; Iszatt et al., 2016). Overall, this case-study estimated thyroid-disrupting activities in Norwegian breast milk to 210 µg DON eq./kg bw/day (antagonistic activity) as well as 453 µg PFOA eq./kg bw/day (competitive activity towards T4 for TTR binding), largely exceeding the current threshold values established by regulatory authorities (Schrenk et al., 2020). Therefore, it is of major interest to conduct target chemical analyses aiming to identify classes of bioaccumulating contaminants causing the thyroid disrupting activities observed in human milk so as to further provide better directives to sensitive populations, including mothers and their nursing child.

Apolar EDCs could interfere with polar EDCs by muting their effects when combined as mixtures

For the purpose of this thesis, we developed a two-step method capable of extracting EDCs from human milk samples. Developing one suitable universal method capable of extracting EDCs with widely different polarity appeared to be very challenging. Thus, we designed a method allowing the extraction of apolar EDCs in one fraction and polar EDCs, including endogenous hormones, in a separate fraction. In addition, to also get a realistic EDC representation for each participant, we reconstituted a mixture derived from the combination of both fractions. Early results indeed showed that assessing apolar and polar fractions separately could bring valuable additional information. Therefore, throughout the present work, apolar EDCs, polar EDCs and the combination of both were assessed separately on CALUX reporter gene assays. As none of the apolar fractions of milk samples were active on the (anti-)AR and (anti-)TRβ bioassays (values <LOQ), results suggested that the anti-androgenic activity as well as anti-TRβ and T4-TTR competitive properties detected in human milk samples could be solely due to the presence of polar anthropogenic EDCs. Further analysis on the anti-AR CALUX demonstrated that reconstituted mixtures overall were significantly less active than the polar fraction itself. These findings highlighted a possible interaction between EDCs of different polarity, whereby apolar extracts acted as potential inhibitors of the polar fractions. On the contrary, anti-TRβ analysis gave the opposite result showing a possible synergy between apolar and polar fractions when combined as a mixture. Overall, these findings suggest a complex mode of action of EDCs on different endocrine endpoints, greatly depending on mixture composition. Results matched with previous studies, reporting that the sole investigation of specific classes of contaminants could lead to misinterpretations by overlooking inhibiting or synergistic effects caused by a potential mixture effect (Kortenkamp,

2014; Rajapakse, Silva, & Kortenkamp, 2002). Overall, this thesis demonstrates the importance of measuring hormonal activity derived from total EDCs content rather than focusing on a more selective subset of chemicals. Through total activity evaluation, bioassay-based monitoring methods such as CALUX reporter gene assays offer a more realistic overview of the potential effects of a biofluid's contamination by anthropogenic toxicants, in comparison with traditional chemical analysis. Nevertheless, it is undeniable that a more comprehensive analysis of mixture composition and EDC combined effects, using both biological- and chemical-based methods, is needed to further elucidate EDC potency and effects on key elements of the endocrine system.

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Supporting Information

Chapter Two: Antagonistic Activity Towards the Androgen Receptor Independent from Natural Sex Hormones in Human Milk Samples from the Norwegian HUMIS Cohort

Appendix A.1

Table 1. Instrumental settings for UHPLC-Q-TOF-MS/MS chemical analysis

Q-TOF	Instrument	maXis 4G equipped with HD collision cell (Bruker, Leiderdorp, the Netherlands)
	Ionization	ESI positive and negative
		Nebulizer gas flow: 1 Bar Dry gas flow: 8.0 l/min Dry heater: 200°C Capillary voltage: 3250V / 3500V (positive/negative) End Plate Offset: -500V
	Funnel RF	250 Vpp
	Multipole RF	250 Vpp
	Collision cell	Cell RF 400 Vpp
		Transfer time: 80 µs Pre pulse storage time: 10 µs
	Scan range	80 – 1200 m/z
	Spectra rate	4 Hz
	Mass calibration	Automatic internal calibration with 2 mM sodium formate in 1:1 water/methanol solution
	MS/MS	AutoMS/MS mode (data-dependent)
	AutoMS/MS settings	Cycle time: 1.0 sec Threshold: 7500 cts Smart exclusion: 5x Active exclusion: Exclude after 3 spectra; Release after 0.50 min; Reconsider Precursor if Current intensity / Previous intensity = 5x CID interpolated list: 100: type=Base; width=8; CE=15 500: type=Base; width=8; CE=35 1000: type=Base; width=10; CE=50 2000: type=Base; width=15; CE=75 Precursor Acquisition Control: Low: 10000 cts; scan at 2 Hz; High: 500000 cts; scan at 8 Hz
UHPLC	Instrument	Nexera (Shimadzu, Den Bosch, the Netherlands)
	Flow, column, mobile phase, column temperature	Phenomenex Kinetex Fluor 5 100x2.1mm 1.7u particle size 0.250mL/min Injection vol. 20 µL
	UV detection	254 nm
	Injection volume	20 µl
	Gradient program	T=0: 20%B; T=25: 100%B; T=28: 100%B; T=28.5: 20%B Equilibration between injections: 10 min First minute diverted to waste

Appendix A.2

Appendix A.2 can be found online at https://doi.org/10.1016/j.envint.2020.105948.

Chapter Three: Anti-Androgenic Compounds in Breast Milk and Possible Association with Cryptorchidism Among Norwegian Boys in the HUMIS Birth Cohort

Supplementary Table 1. Profile of study participants for anti-AR case-control study compared to the eligible and entire cohort.

	Enrolled in HUMIS cohort	Eligible (mother-son) Cohort	Case-control study participants
	N=2,606	N=1,262	N=199
Maternal age (years)	30 (27-33)	30 (26-33)	30 (26-33)
Maternal Education level			
Low	223 (8.6%)	113 (9.0%)	19 (9.5%)
Middle	319 (12.2%)	158 (12.5%)	26 (13.1%)
High	1,952 (74.9%)	970 (76.9%)	150 (75.4%)
Missing	112 (4.3%)	21 (1.7%)	4 (2.0%)
Birth weight			
<2500	178 (6.8%)	56 (4.4%)	5 (2.5%)
2500-4000	1,853 (71.1%)	876 (69.4%)	146 (73.4%)
> 4000	574 (22.0%)	329 (26.1%)	48 (24.1%)
Missing	1 (0.0%)	1 (0.1%)	
Parity	1,063 (42.5%)	531 (43.6%)	78 (40.0%)
Gestational age (days)	281 (273-288)	282 (274-288)	281.5 (273-288)
Small for gestational age	261 (10.0%)	113 (9.0%)	19 (9.5%)
Preterm	242 (9.3%)	101 (8.0%)	13 (6.5%)
Caesarean section	434 (16.7%)	205 (16.2%)	27 (13.6%)
Pre-pregnancy BMI			
Under weight	80 (3.1%)	39 (3.1%)	4 (2.0%)
Normal	1,536 (58.9%)	750 (59.4%)	125 (62.8%)
Overweight	565 (21.7%)	280 (22.2%)	39 (19.6%)
Obese	260 (10.0%)	127 (10.1%)	22 (11.1%)
Missing	165 (6.3%)	66 (5.2%)	9 (4.5%)
Smoking in pregnancy			
never smoking	1,557 (59.7%)	747 (59.2%)	132 (66.3%)
past smoker	682 (26.2%)	342 (27.1%)	52 (26.1%)
occasional	63 (2.4%)	35 (2.8%)	3 (1.5%)
daily smoker LE 10	208 (8.0%)	97 (7.7%)	8 (4.0%)
daily smoker >10	32 (1.2%)	17 (1.3%)	2 (1.0%)
Missing	64 (2.5%)	24 (1.9%)	2 (1.0%)
Preeclampsia	115 (4.4%)	55 (4.4%)	7 (3.5%)
Gestational diabetes	15 (0.6%)	9 (0.7%)	1 (0.5%)

Supplementary Table 2. Missing-value patterns of the data.

	Pattern								
Percent	1	2	3	4	5	6	7	8	9
17	1	1	1	1	1	1	1	1	1
41	1	1	1	1	1	1	1	0	0
23	1	1	1	1	1	1	1	1	0
11	1	1	1	1	1	1	1	0	1
2	1	1	1	1	1	1	0	1	0
1	1	1	1	0	1	1	1	1	0
1	1	1	1	1	1	1	0	0	0
<1	0	1	1	1	1	1	0	1	0
<1	1	0	1	1	0	0	0	0	0
<1	1	0	1	1	1	1	1	0	0
<1	1	1	0	1	1	1	1	0	1
<1	1	1	0	1	1	1	1	1	0
<1	1	1	0	1	1	1	1	1	1
<1	1	1	1	0	0	0	0	0	0
<1	1	1	1	0	1	1	1	0	0
<1	1	1	1	1	0	0	0	0	0
<1	1	1	1	1	0	0	1	0	0
<1	1	1	1	1	1	0	1	0	0
100									

Note: 17% of the data is complete for all variables while 83% are missing for either one or more of the variables.
1. Gestational age 2. Smoking 3. Fat content percent 4. Maternal education 5. Number of siblings 6. Antibiotics use during pregnancy 7. Pre-pregnancy BMI 8. PCB194 9. Alcohol consumption.

However, the 83% missing was reduced to 7% when variables with high missing (alcohol use, smoking, antibiotics, maternal toxicants, and fat content) were removed after Sensitivity analysis showed that including them in the model didn't change the result (See below Supp. Figure 1-5). Therefore, after removing these variables, 93% had complete data for the rest of the variables.

Supplemental Table S3. Unadjusted and adjusted estimates from logistic regression for the associations between anti-AR activity from different fractions (mixture, polar, apolar) and the risk of cryptorchidism among cases (n=94) and controls (n=105) in HUMIS cohort.

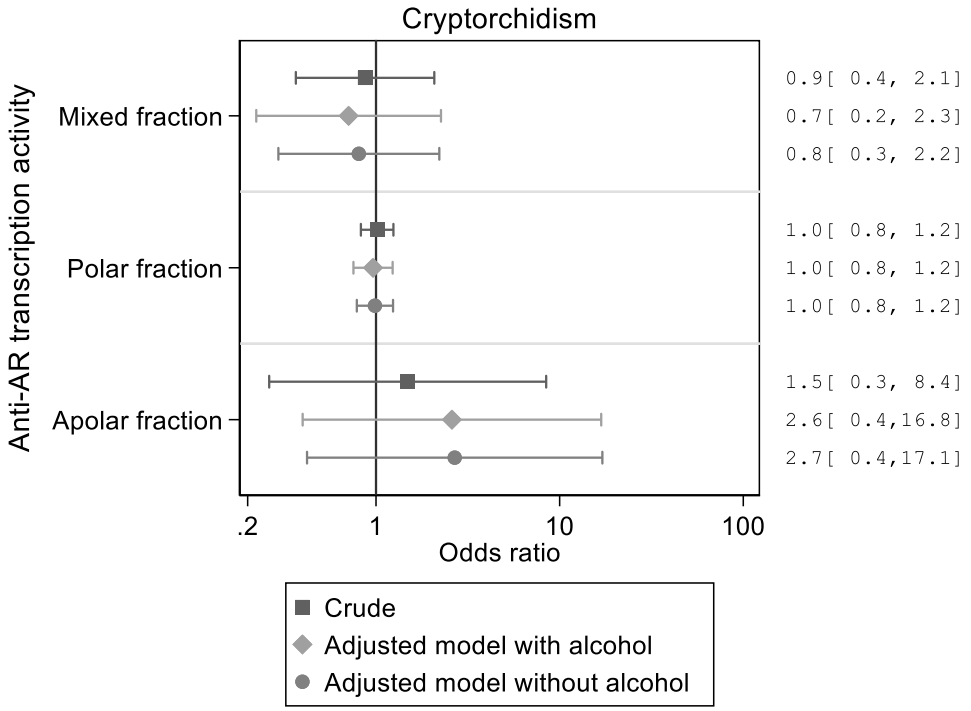
	Crude				Adjusted[a]				Further adjusted[b]			
	OR	**95% CI**		***p-value***	**OR**	**95% CI**		***p-value***	**OR**	**95% CI**		***p-value***
Cumulative cryptorchidism												
Mixed fraction	0.87	0.36	2.07	*0.75*	0.65	0.25	1.71	*0.39*	0.59	0.22	1.61	*0.31*
Polar fraction	1.01	0.82	1.24	*0.89*	1.00	0.80	1.24	*0.99*	1.00	0.80	1.25	*0.97*
Apolar fraction	1.49	0.26	8.44	*0.65*	1.77	0.30	10.39	*0.53*	2.18	0.36	2.58	*13.3*

Note: [a]Minimal adjusted model: Maternal age, socio economic status, Pre-pregnancy BMI, gestational age and parity

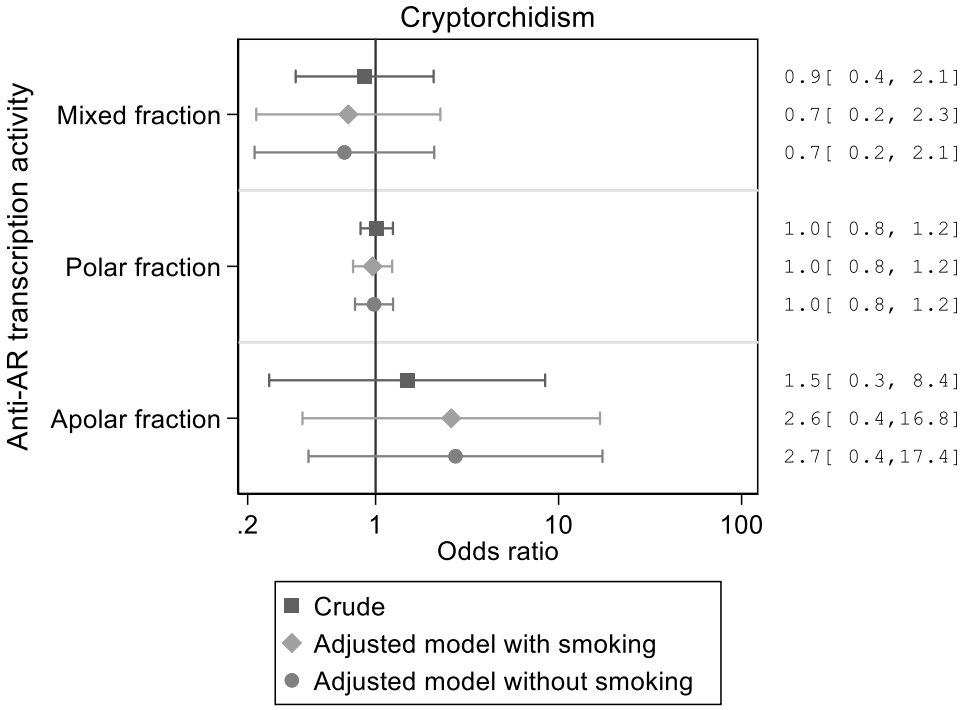
[b]Fully adjusted model: Additional adjustment including gestational diabetes and preeclampsia.

OR: Odd Ratio; 95% CI: 95% Confidence interval

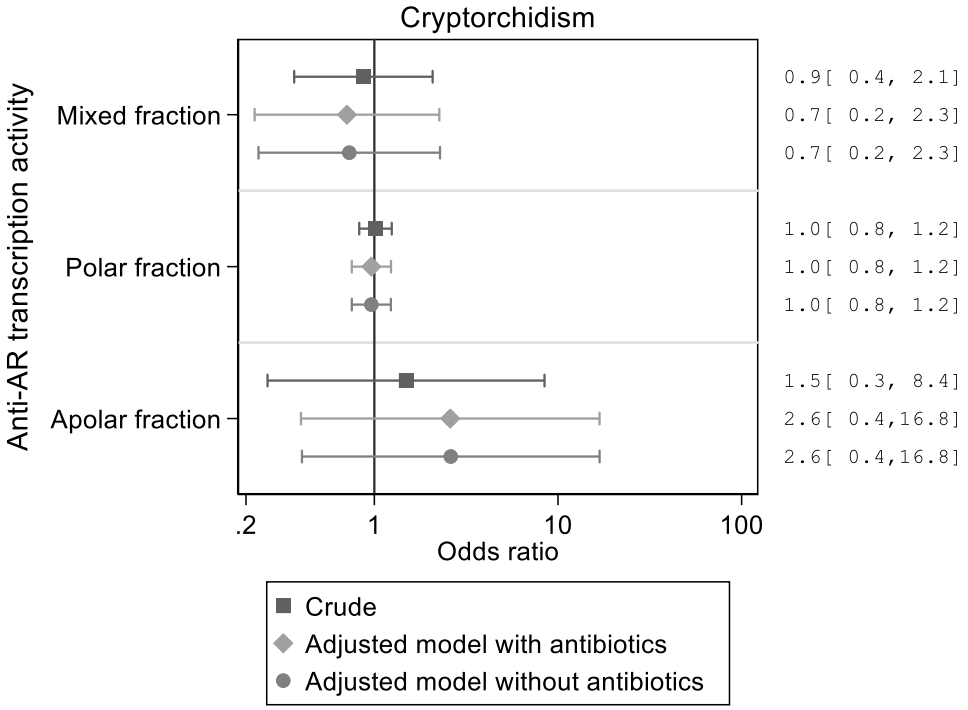
Sensitivity Analysis was performed on the multiple imputed data set to check if including some of the variables (***alcohol use, smoking, antibiotics, maternal toxicants, and fat content***) would lead to changes in the result.



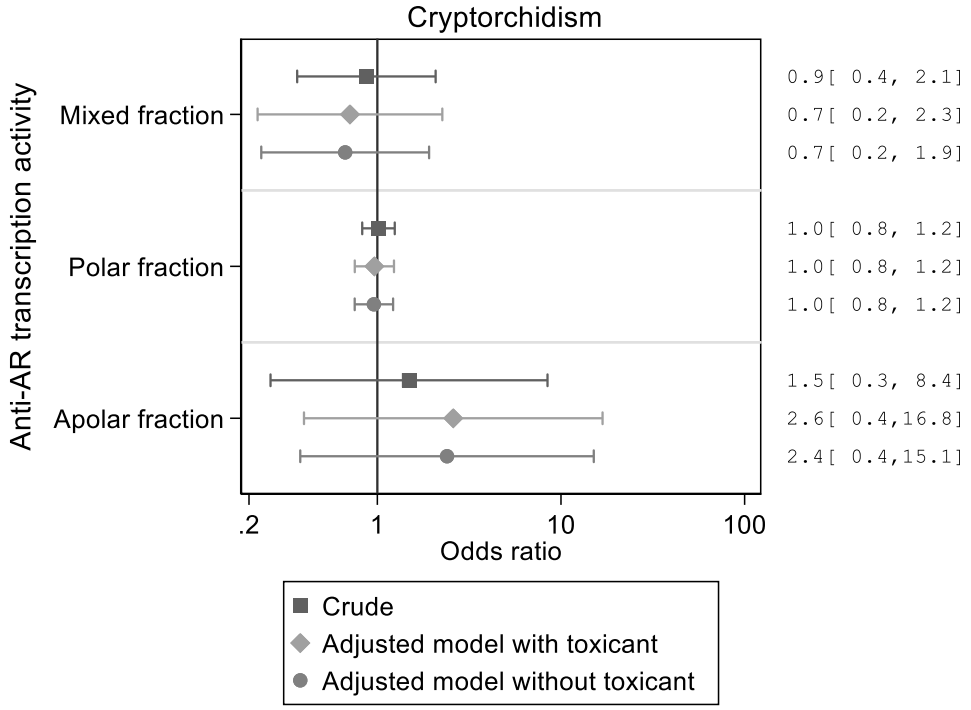
Supplemental Figure 1. Sensitivity analysis for alcohol use in pregnancy



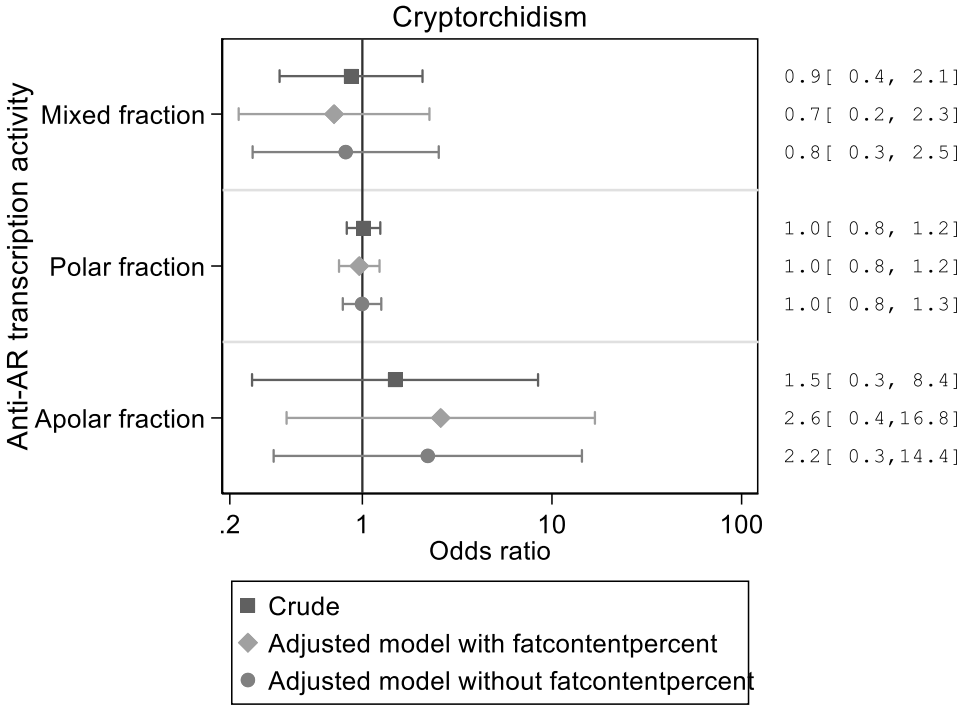
Supplemental Figure 2. Sensitivity analysis for smoking in pregnancy



Supplemental Figure 3. Sensitivity analysis for antibiotic use in pregnancy



Supplemental Figure 4. Sensitivity analysis for selected maternal toxicant in pregnancy



Supplemental Figure 5. Sensitivity analysis for fat content percentage of breast milk in pregnancy

Chapter Four: Evaluation of A Panel of In Vitro Methods for Assessing Thyroid Receptor B and Transthyretin Transporter Disrupting Activities

Annex 1

Table 1. Cytotoxicity results of test compounds for TRβ CALUX evaluation, agonistic mode.

Compound	Minimum (M)	Maximum (M)	Cytotoxicity (M)
1-850	1.0E-12	1.0E-05	≥1.0E-05
2-AAF	1.0E-12	1.0E-05	-
aflatoxin b1	3.0E-12	3.0E-05	-
amiodarone	3.0E-13	3.0E-06	-
at-RA	3.0E-12	3.0E-05	≥3.0E-05
BPA	3.0E-12	3.0E-05	-
dieldrin	3.0E-12	3.0E-05	-
endosulfan	3.0E-12	3.0E-05	-
hydroquinone	3.0E-12	3.0E-05	-
methoxyacetic acid	3.0E-12	3.0E-05	-
T2-toxin	1.0E-01	1.0E-04	≥1E-7
T3	1.0E-12	1.0E-05	-
T4	1.0E-12	1.0E-05	-
TBBPA	1.0E-12	1.0E-05	≥1.0E-05
TETRAC	1.0E-12	1.0E-05	-
TRIAC	1.0E-11	1.0E-04	≥1.0E-04
valproic acid	1.0E-12	1.0E-05	-
vinclozolin	1.0E-12	1.0E-05	-

Note: Concentration range and cytotoxicity results for all test compounds analyzed in the TRβ CALUX. Cytotoxicity was determined by Cytotox CALUX assay, background control and/or visual observation.

Table 2. Cytotoxicity results of test compounds for TRβ CALUX evaluation, antagonistic mode.

Compound	Minimum (M)	Maximum (M)	Cytotoxicity (M)
1-850	1.0E-12	1.0E-05	-
4-nonylphenol	3.0E-12	3.0E-05	≥3.0E-05
aflatoxin b1	3.0E-12	3.0E-05	-
amiodarone	3.0E-12	3.0E-06	-
at-RA	3.0E-12	3.0E-05	-
bisphenol A	3.0E-12	3.0E-05	-
deoxynivalenol	1.0E-09	3.0E-06	-
dibutylphthalate	3.0E-12	3.0E-05	-
dronedarone	1.0E-12	1.0E-05	≥1.0E-05
endosulfan	3.0E-12	3.0E-05	≥3e-6
pinoresinol	3.0E-12	3.0E-05	-
T2-toxin	1.0E-11	1.0E-04	≥1e-5
T3	1.0E-12	1.0E-05	-
TBBPA	1.0E-12	1.0E-05	-
zearalenone	1.0E-11	1.0E-04	≥1e-5

Note: Concentration range and cytotoxicity results for all test compounds analyzed in the anti-TRβ CALUX. Cytoxicitiy was determined by Cytotox CALUX assay, background control and/or visual observation.

Table 3. Cytotoxicity results of test compounds for TTR-TRβ CALUX evaluation.

Compound	Minimum (M)	Maximum (M)	Cytotoxicity (M)
4-nonyphenol	1.1E-07	3.2E-04	-
alachlor	1.1E-07	3.2E-04	-
BPA	1.1E-06	3.2E-03	-
DES	1.1E-08	3.2E-05	-
PBP	1.1E-09	3.2E-06	-
PCP	1.1E-07	3.2E-04	-
PFOA	1.1E-08	3.2E-05	-
PFOS	1.1E-10	3.2E-06	-
TCBPA	1.1E-09	3.2E-06	-
TBBPA	1.1E-09	3.2E-06	-
t2-toxin	1.1E-09	3.2E-06	-

Note: Concentration range and cytotoxicity results for all test compounds analyzed in the TTRTRβ CALUX. Cytotoxicity was determined by background control and visual observation.

Annex 2

Table 1. Selection of active and non-active compounds for TRβ CALUX evaluation, agonistic mode.

Compound	CAS number	Reported activity	Reference
1-850	251310-57-3	Non-active	Schapira *et al.,* 2003 [1]
2-AAF	53-96-3	Active	BDS internal database
aflatoxin B1	1162-65-8	Non-active	BDS internal database
amiodarone	96027-74-6	Non-active	Drvota *et al.,* 1995 [2]
at-RA	302-79-4	Active	BDS internal database
BPA	80-05-7	Non-active	Sun *et al.,* 2009 [3]
dieldrin	60-57-1	Non-active	BDS internal database
endosulfan	959-98-8	Non-active	ToxCast [4]
hydroquinone	123-31-9	Unknown	BDS internal database
methoxyacetic acid	625-45-6	Non-active	BDS internal database
T2-toxin	21259-20-1	Unknown	BDS internal database
T3	5714-08-9	Active	Cheek *et al.,* 1999 [5]
T4	51-48-9	Active	Cheek *et al.,* 1999
TBBPA	79-94-7	Non-active	Sun *et al.,* 2009
TETRAC	67-30-1	Active	Cheek *et al.,* 1999
TRIAC	51-24-1	Active	Cheek *et al.,* 1999
valproic acid	99-66-1	Non-active	BDS internal database
vinclozolin	50471-44-8	Non-active	BDS internal database

Note: All compounds were selected based on either available data, prior intra-laboratory studies or chemical properties. In italics, the reference compound T3. Compounds referred as "BDS internal database" will not be used for predictivity calculations. Report to 2.3 for chemicals abbreviation. All compounds were purchased from Sigma Aldrich with the exception of 2-AAF and 1-850 which were obtained from Ultra Scientific and Cayman, respectively.

Table 2. Selection of active and non-active compounds for TRβ CALUX evaluation, antagonistic mode.

Compound	CAS number	Reported activity	Reference
1-850	251310-57-3	Active	Schapira *et al.,* 2003
4-nonylphenol	104-40-5	Active	ToxCast [5]
aflatoxin B1	1162-65-8	Non-active	BDS internal database
amiodarone	96027-74-6	Non-active	Drvota *et al.,* 1995
At-RA	302-79-4	Unknown	BDS internal database
BPA	80-05-7	Active	Sun *et al.,* 2009
deoxynivalenol	4330-21-6	Active	Demaegdt *et al.,* 2016 [5]
dibutylphthalate	84-74-2	Active	Shen *et al.,* 2009 2016 [7]
dronedarone	141626-36-0	Non-active	Droggrell & Hancox, 2004 2016 [8]
endosulfan	959-98-8	Active	ToxCast
pinoresinol	487-36-5	Active	Ogungbe *et al.,* 2014 2016 [9]
T2-toxin	21259-20-1	Active	BDS internal database
T3	5714-08-9	Non-active	Cheek *et al.,* 1999
TBBPA	79-94-7	Active	Sun *et al.,* 2009
zearalenone	17924-92-4	Active	Kiss *et al.,* 2018 [10]

Note: All compounds were selected based on either available data, prior intra-laboratory studies or chemical properties. In italics, the reference compound deoxynivalenol. Compounds referred as "BDS internal database" will not be used for predictivity calculations. Report to 2.3 for chemicals abbreviation. All compounds were purchased from Sigma Aldrich with the exception of 1-850 which was obtained from Cayman.

Table 3. Selection of active and non-active compounds for TTR-TRβ CALUX evaluation.

Compound	CAS number	Reported activity	Reference
4-nonyphenol	25154-52-3	Active	Simon *et al.,* 2013 [11]
alachlor	15972-60-8	Non-active	Cheek *et al.,* 1999
BPA	80-05-7	Active	Cao *et al.,* 2011 [12]
DES	56-53-1	Active	Ishihara *et al.,* 2003 [13]
PBP	608-71-9	Active	Meerts *et al.,* 2000 [14]
PCP	87-86-5	Active	Van den Berg, 1990 [15]
PFOA	335-67-1	Active	Weiss *et al.,* 2009 [16]
PFOS	1763-23-1	Active	Weiss *et al.,* 2009
T2-toxin	21259-20-1	Unknown	BDS internal database
TBBPA	79-94-7	Active	Meerts *et al.,* 2000
TCBPA	79-95-8	Active	Meerts *et al.,* 2000

Note: All compounds were selected based on either available data, prior intra-laboratory studies or chemical properties. In italics, the reference compound TBBPA. Compounds referred as "BDS internal database" will not be used for predictivity calculations. Report to 2.3 for chemicals abbreviation. All compounds were purchased from Sigma Aldrich with the exception of PFOS which was obtained from Chemika.

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Samenvatting

Dit proefschrift onderzoekt de aanwezigheid en potentiële effecten van hormoonverstorende chemicaliën (EDC) in humane melk, gebruikmakend van monsters uit het Noorse HUMIS cohort. Deze studie is gebaseerd op een tweestapsbenadering, waarbij als eerste stap de extractie van hormoon actieve stoffen plaatsvindt gebaseerd op hun polariteit. Als tweede stap worden de apolaire- en polaire extracten en een combinatie van beide geanalyseerd met behulp van *in vitro* humaan-gebaseerde reportergen CALUX bioassays die belangrijke eindpunten van het endocriene systeem omvatten: oestrogenen, androgenen en schildklierhormonen.

Hoofdstuk één geeft achtergrond over contaminanten met hormoonverstorende eigenschappen en introduceert de steroïde- en schildklier signaleringsroutes. Dit hoofdstuk beschrijft ook eerdere bevindingen van het Noorse HUMIS geboorte-cohort en de belangrijkste doelstellingen van dit proefschrift. Bovendien worden, gezien hun belang in de studies die in dit proefschrift worden gepresenteerd, effect-gebaseerde bioassays en effect-gestuurde fractionering geïntroduceerd.

Hoofdstuk twee beschrijft een teststudie uitgevoerd op tien humane melkmonsters gebruik makend van de (anti-)ERβ en (anti-) AR CALUX bioassays. Hoewel er nagenoeg geen oestrogene activiteit werd gedetecteerd, geeft deze studie aan dat alle monsters behalve één kwantificeerbare hoeveelheden anti-androgene activiteit van onbekende oorsprong bevatten. Interessant is dat de metingen een hogere activiteit lieten zien in de polaire fracties in vergelijking met de gereconstitueerde apolaire-polaire extracten, terwijl geen van de apolaire fracties actief was in de anti-AR CALUX bioassay. Verder onderzoek met behulp van effect-gestuurde fractionering en uitsluitingsmethoden toonde aan dat de waargenomen anti-androgene activiteit niet verklaard kon worden door de aanwezigheid van natuurlijke hormonen (androstendion, DHEA, E1, E2, pregnolon, progesteron, testosteron) en/of metabolieten daarvan. Deze resultaten suggereren daarom dat het meer waarschijnlijk is dat de waargenomen anti-AR activiteit in humane melkmonsters wordt veroorzaakt door nog niet geïdentificeerde polaire antropogene contaminanten.

Er is toenemend *in vitro* en *in vivo* bewijs dat suggereert dat vroegtijdige blootstelling aan anti-androgenen kan resulteren in reproductieve stoornissen, zoals cryptorchisme. Om die reden werden er, in **Hoofdstuk drie**, 199 humane melkmonsters uit het HUMIS cohort geselecteerd voor een casestudy, inclusief een subset van 94 moeders die bevallen zijn van jongens met niet-ingedaalde testikels en een subset van 105 moeders 'controle' die bevallen zijn van jongens zonder cryptorchisme. In dit hoofdstuk werd een potentieel verband tussen anti-androgene activiteit, gemeten met behulp van anti-AR CALUX in moedermelk, en cryptorchisme geëvalueerd. Hoewel statistische analyse geen significant verschil liet zien tussen de op humane melk gebaseerde anti-AR activiteit in de controle- en cryptorchisme monsters, kon een mogelijk verband tussen de aanwezigheid van apolaire verbindingen in humane melk en een prevalentie van niet-ingedaalde testikels niet worden uitgesloten. Over het algemeen werd er een anti-androgene activiteit in het merendeel van de melkmonsters gemeten. Analyse van fracties met verschillende polariteit, d.w.z. apolaire, polaire en gecombineerde extracten, bevestigde de eerdere bevindingen uit hoofdstuk twee, en werd benadrukt dat polaire verbindingen het meest bijdragen aan de anti-androgene activiteit in moedermelk. Hoewel de herkomst van zulke verbindingen nog steeds onduidelijk is laat deze studie het belang zien van het beoordelen van mengsels in plaats van individuele chemicaliën of subklassen.

Hoewel gebleken is dat geslachtshormonen bijzonder gevoelige doelwitten zijn van hormoonverstoring in het vroege levensstadium zijn er in de afgelopen jaren ook zorgen gerezen over de invloed van hormoonverstorende stoffen op het schildklierstelsel door onder meer veranderingen in de circulerende schildklierhormoonspiegel te veroorzaken en te interfereren met het serumtransport van het schildklierhormoon, in het bijzonder transthyretin (TTR). In **Hoofdstuk vier**, werd de ontwikkeling en validatie van een nieuw schildklier CALUX panel geëvalueerd, specifiek ontworpen om het vermogen tot interactie van hormoonverstorende stoffen met de schildklierreceptor β (TRβ) (TRβ CALUX assay) en/of TTR (TTR-TRβ CALUX assay) te beoordelen. De TRβ CALUX assay bestaat uit een U2OS osteosarcoomcellijn die endogeen TRβ tot expressie brengt en stabiel is getransfecteerd met een aan schildklier responsieve elementen gekoppelde luciferase reportergen. Deze TRβ CALUX assay is in staat om de op schildklierhormoonreceptor gebaseerde agonistische activiteit te beoordelen door middel van het kwantificeren van de geproduceerde luciferase activiteit. TRβ CALUX assay kan ook gebruikt worden voor het meten van de antagonistische activiteit door middel van pre-stimulatie van de cellen met natuurlijk ligand T3 (anti-TRβ CALUX assay) of voor de evaluatie van de T4-TTR bindingscompetitie in combinatie met de TTR bioassay. Elke bioassay (TRβ and TTR-TRβ) werd gevalideerd met behulp van een reeks bekende positief en negatief schildklierverstorende stoffen en vertoonde een zeer goede voorspelbaarheid wat suggereert dat het nieuw ontwikkelde CALUX panel een krachtig hulpmiddel kan zijn bij het voorspellen van de effecten van hormoonverstorende stoffen op de endogene schildklierhormoon werking.

In **Hoofdstuk vijf**, werd het nieuw ontwikkelde schildklier-specifieke CALUX assay panel gebruikt om een set van per- en polyfluoralkyl verbindingen te onderzoeken, waarvan verondersteld werd dat ze mogelijk schildklierverstoorders zijn. Geen van de geteste PFAS vertoonde een agonistische activiteit in de TRβ CALUX reportergen assay, terwijl zeven PFAS antagonistische activiteit vertoonden op TRβ. TTR- TRβ CALUX analyses toonde aan dat alle PFAS de eigenschappen hebben om de competitie aan te gaan met T4 bij de TTR binding, zij het met verschillende potentie. In dit hoofdstuk, hebben we ook onderzocht of humane melkmonsters uit het Noorse HUMIS cohort schildklierhormoon verstorende activiteit bevat die mogelijk verband houdt met de aanwezigheid van contaminanten, waaronder PFAS. Hoewel er geen TRβ agonistische activiteit werd gedetecteerd vertoonden de meeste moedermelk extracten wel antagonistische activiteit en alle behalve één vertoonden een competitieve T4-TTR bindingspotentie van 0.41 tot 9.1 µg PFOA eq./g melk. Gezien hun vermogen de competitie aan te gaan met het schildklierhormoon bij het TTR transport en TRβ activatie tegen te gaan suggereren de in hoofdstuk vijf gepresenteerde resultaten dat PFAS potente schildklierhormoon verstoorders kunnen zijn. Hoewel het aantal monsters vrij klein was (tien) toonde de teststudie uitgevoerd op de HUMIS monsters de aanwezigheid van schildklierhormoon verstorende activiteit aan in extracten van moedermelk. Verdere berekeningen gebaseerd op PFOA (perfluoroctaanzuur) en PFOS (perfluorsulfonaat) monsterconcentraties toonden aan dat slechts een klein percentage van de TRβ antagonistische activiteit verklaard kon worden door PFAS contaminatie, wat kan duiden op de aanwezigheid van andere onbekende schildklierhormoon verstorende chemicaliën in humane melk.

Résumé

Fondée sur des échantillons de lait provenant de la cohorte norvégienne HUMIS, cette thèse étudie la présence de perturbateurs endocriniens (PEs) dans le lait maternel ainsi que leurs potentiels effets sur le développement du nourrisson. Les différents chapitres de ce projet sont basés sur une approche en deux étapes permettant dans un premier temps de préparer les échantillons en isolant leurs composés chimiques actifs en fonction de leur polarité. Dans un second temps, les extraits apolaires, polaires et leur combinaison (mélange) découlant de cette extraction sont étudiés à l'aide de méthodes d'analyse *in vitro* reposant sur l'utilisation de lignées cellulaires humaines. Ces essais biologiques dits 'CALUX' permettent l'étude des principaux acteurs du système endocrinien : les œstrogènes, les androgènes et les hormones thyroïdiennes (HTs).

Le **premier chapitre** contextualise notre sujet en introduisant les éléments majeurs nécessaires à la compréhension du projet à savoir la notion de perturbateurs endocriniens, les voies de signalisation stéroïdienne et thyroïdienne, ainsi que les méthodes d'analyses utilisées tout au long de l'étude (essais biologiques et fractionnement dirigé). Ce chapitre présente également les résultats découlant de précédentes études sur la cohorte HUMIS et détaille les principaux objectifs de cette thèse.

Le deuxième chapitre constitue une étude pilote réalisée sur dix échantillons de lait maternel à l'aide de deux essais biologiques CALUX : récepteur oestrogénique beta (ERβ) CALUX et récepteur androgénique (AR) CALUX. Bien que presqu'aucune activité œstrogénique n'ait été détectée, ce travail montre que tous les échantillons sauf un présentent une activité anti-androgénique (anti-AR) d'origine inconnue. Aucune fraction apolaire testée n'a été interprétée comme active. Fait intéressant, les résultats démontrent que ce type d'activité est plus élevée dans les fractions polaires que dans les mélanges avec les extraits apolaires supposant une potentielle interaction entre les deux types de composés. Une étude plus approfondie impliquant des méthodes de fractionnement et d'exclusion dirigés montre que l'activité anti-androgénique observée dans les échantillons ne pouvait pas s'expliquer par la présence d'hormones naturelles (androstènedione, DHEA, E1, E2, pregnenolone, progestérone, testostérone) et/ou de métabolites associés. Par conséquent, ces résultats suggèrent que l'activité anti-AR présente dans les échantillons de lait maternel est plus susceptible d'être causée par des contaminants anthropiques polaires encore non identifiés que par des composés présents naturellement dans le corps.

De plus en plus de preuves *in vitro* et *in vivo* suggèrent qu'une exposition précoce à des anti-androgènes peut s'avérer néfaste pour le bon développement et fonctionnement de l'appareil reproducteur masculin, pouvant engendrer des malformations congénitales telles que la cryptorchidie i.e. défaut de migration du testicule. Par conséquent, 199 échantillons de lait maternel provenant de 94 mères ayant donné naissance à des garçons avec des testicules non-descendus ainsi que 105 mères dites « contrôles », c'est-à-dire ayant donné naissance à des garçons sans cryptorchidie, ont été sélectionnés pour effectuer une étude de cas présentée dans le **troisième chapitre**. Dans ce chapitre, nous évaluons la possible relation entre l'activité anti-androgène détectée dans le lait maternel, et le risque de cryptorchidie chez le nouveau-né. Dans l'ensemble, une activité anti-androgénique a été mesurée dans la majorité des échantillons de lait. Bien que l'analyse statistique n'ait montré aucune différence significative entre l'activité mesurée dans les échantillons témoins et celle dans les échantillons dits 'cas', nous avons observé une éventuelle association entre la présence de composés apolaires dans le lait maternel et le risque de cryptorchidie. L'analyse des différentes fractions, c'est-à-dire des extraits apolaires, polaires et combinés, a confirmé les résultats rapportés dans le chapitre deux, soulignant que les composés polaires seraient en grande majorité responsable de l'activité anti-AR présente dans le lait maternel. Bien que les origines

de ces composés ne soient pas encore claires, cette étude démontre l'importance d'évaluer les mélanges de PEs dans leur ensemble plutôt que d'étudier les composés individuellement.

Bien que les stéroïdes soient une cible particulièrement prisée des PEs, ces dernières années ont mis en lumière la capacité des PEs à cibler le système thyroïdien, causant entre autres des modifications des niveaux des HTs circulantes ainsi que des interférences avec leurs transporteurs, en particulier la protéine de transport transthyrétine (TTR). Ainsi, un nouveau panel d'essais biologiques CALUX spécialement conçus pour évaluer les capacités des PEs à interagir avec le récepteur thyroïdien β (TRβ) (TRβ CALUX) ou avec le transporteur TTR (TTR-TRβ CALUX) a été développé et validé dans le **quatrième chapitre** de cette thèse. Le TRβ CALUX est basé sur une lignée cellulaire U2OS exprimant TRβ et stablement transfectée avec le gène rapporteur de la luciférase, lié aux éléments de réponse thyroïdiens. Grâce à ce gène rapporteur, il est possible de quantifier l'activité des PEs sur TRβ en mesurant la quantité de lumière produite. Il est également possible de mesurer l'activité dite 'antagoniste' des PEs en pré-stimulant les cellules avec le ligand naturel T3 ainsi que d'évaluer leur capacité à entrer en compétition avec T4 pour leur transport via TTR. Chaque essai (TRβ et TTR-TRβ CALUX), a été validé à l'aide d'une gamme de composés positifs connus pour être des perturbateurs thyroïdiens, ainsi que des substances voulues négatives. Cette évaluation a montré une très bonne prédictivité suggérant que ce nouveau panel CALUX peut être un outil puissant dans la prédiction des effets des PEs sur le système thyroïdien.

Dans le **cinquième chapitre**, le panel thyroïdien CALUX a été utilisé pour étudier treize substances per- et poly-fluoroalkylées (PFAS), possibles perturbateurs thyroïdiens. Aucun des PFAS testés n'a engendré l'activation du TRβ cependant, sept PFAS ont été identifiés comme antagonistes du récepteur. L'analyse TTR-TRβ CALUX a montré que tous les PFAS testés peuvent entrer en compétition avec le ligand naturel T4 pour liaison avec TTR, bien que leur niveau d'activité soit dépendant de leur structure chimique. Dans ce chapitre, dix échantillons de lait maternel ont également été analysés sur le panel thyroïdien. Bien qu'aucune activité agonistique n'ait été détectée, la majorité des extraits ont montré une activité antagoniste envers TRβ et tous, sauf un, ont été actifs sur le TTR- TRβ CALUX ce qui équivaut à une activité de 0,41 à 9,1 µg PFOA équivalent/g de lait. Compte tenu de leurs capacités à rivaliser avec les HTs pour le transport TTR et à inhiber TRβ, les résultats présentés dans le chapitre cinq suggèrent que les PFAS peuvent être de puissants perturbateurs thyroïdiens. Bien que la taille de l'échantillon soit relativement limitée, cette étude pilote démontre la présence d'une activité anti-thyroïdienne dans le lait maternel. De plus amples calculs basés sur les concentrations de PFOA et PFOS présentes dans les échantillons de la cohorte HUMIS ont démontré que seul un faible pourcentage de l'activité antagoniste pouvait s'expliquer par une contamination aux PFAS, suggérant la présence d'autres produits chimiques inconnus dans le lait maternel.

Curriculum Vitae

Bérénice Constance Collet was born on July 23rd 1992 in Charleville-Mézières, Ardennes, France. She graduated of a bachelor's degree Cellular Biology and Physiology in 2014 from the University of Sciences and Technology of Lille, France. Two years later, she received her master's degree with a specialisation in Biotechnologies: Cellular and Molecular Engineering. The theoretical courses of this MSc were completed by workshops, projects and eleven months of internship spread over a two-year program. In 2015, Bérénice chose to do her internship within Dr. Ina Dobrinski's laboratory at the Department of Comparative Biology and Experimental Medicine of the University of Calgary, Canada. The aim of the project was to differentiate induced pluripotent stem cells into male germline cells in order to complete spermatogenesis. This work was presented as a potential alternative treatment for infertility. In 2016, she dedicated the last part of her MSc to the study of the consequences of an *in utero* foetal exposure to chemicals (genistein and DEHP) on the male reproductive system. Her master's thesis was supervised by Dr. Martine Culty at the Research Institute of the McGill University Health Centre (Montreal, Canada). This two-step work experience allowed her to study both potential origins of reproductive disorders and possible treatments for infertile couples. In 2017, she joined the Marie Curie-Skłodowska Innovative Training Network ProtectED (http://protected.eu.com) and started a PhD project at the Vrije Universiteit in Amsterdam hosted by BioDetection Systems b.v. (Amsterdam, the Netherlands). Supervised by Pr. Dr. Brouwer and Dr. Van der Burg, she investigates endocrine disrupting chemical mixtures in breast milk and possible health consequences for human infants using effect-based methods.

List of Publications

Collet B, Simon E, Van der Linden S, el Abdellaoui N, Naderman M, Man H, Middelhof I, Van der Burg B, Besselink H, Brouwer A. 2019. Evaluation of a panel of in vitro methods for assessing thyroid receptor β and transthyretin transporter disrupting activities. Reprod Toxicol 1–13; doi:10.1016/j.reprotox.2019.05.011.

Collet B, Van Vugt-lussenburg BMA, Swart K, Helmus R, Naderman M, De Rijke E, Eggesbø M, Brouwer A, Van der Burg B. 2020. Antagonistic activity towards the androgen receptor independent from natural sex hormones in human milk samples from the Norwegian HUMIS cohort. Environ Int 143:105948; doi:10.1016/j.envint.2020.105948.

Walker C, Ghazisaeidi S, Collet B, Boisvert A, Culty M. 2020. In-utero Exposure to Low Doses of Genistein and Di-(2-ethylhexyl) Phthalate (DEHP) Alters Innate Immune Cells in Neonatal and Adult Rat Testis. Andrology 1–22; doi:10.1111/andr.12840.

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